Jordan-Burrows Textbook of

BACTERIOLOGY

By WILLIAM BURROWS, Ph.D.

Professor of Bacteriology, Department of Bacteriology
and Parasitology, the University of Chicago

With the Collaboration of

FRANCIS BYRON GORDON, Ph.D., M.D.

Biological Department, Chemical Corps, Camp Detrick,
Maryland; formerly Professor of Bacteriology, Department
of Bacteriology and Parasitology, the University of Chicago

RICHARD JANVIER PORTER, Ph.D.

Associate Professor of Parasitology, School of Public
Health, the University of Michigan

JAMES WILLIAM MOULDER, Ph.D.

Assistant Professor of Biochemistry, Department of
Bacteriology and Parasitology, the University of Chicago

Fifteenth Edition

W. B. SAUNDERS COMPANY

PREFACE

About twenty years ago it was freely predicted by a number of eminent workers that a new era of bacteriology was beginning which would be characterized by a greater emphasis upon the nature of these microorganisms, and especially their biochemical physiology. This has been more than amply confirmed in recent years, and it is becoming increasingly clear that bacteriology has largely emerged from a naturalist period of essentially empirical observation into an analytical, quantitative phase in which the proof of specific bacterial etiology of infectious disease, for example, has been extended into analysis of the functional mechanisms of pathogenesis. The changing approach has given great promise, and the extent to which a book of this kind becomes appreciably obsolete within a few short years is impressive evidence of the progress that has resulted from it. Most encouraging is the extent to which the various applied branches of bacteriology are drawing together on a common fundamental basis, and the relating of these forms to other organisms through basic physiological processes, such as respiration, nutrition and genetic control.

The pattern is reflected in the development of this book through successive editions; its basic premise has been the assumption that the sound pedagogic approach is that of an understanding of fundamental principles out of which application flows. There are few better illustrations of the importance of apparently impractical "pure" research than the elucidation of the nutritive requirements of bacteria, with subsequent development of a rational theory of chemotherapy. This area of research further points up the significance of the anabolic phase of bacterial metabolism as accumulating evidence suggests more and more strongly that the point of attack of certain of the antibacterial drugs is in the reactions of synthesis. In this same vein, the apparently academic question of the relative importance of, on the one hand, selection of chance mutants, and, on the other, a mass action basis of adaptive enzyme formation in the development of drug-fast strains of pathogenic bacteria may, perhaps, prove to be of considerable significance through mutually exclusive adaptations. Considerations such as these tend to reinforce belief in the validity of the premise, and it continues as the basis of the present edition.

There has been, necessarily, a considerable amount of rewriting which has ranged in extent from entire chapters to sections, together with appropriate modification of innumerable minor points. One change in order of presentation has been made with a shift of the chapter on bacterial physiology so that it precedes that on physical and chemical agents.

The chapter on laboratory methods has been completely rewritten in a much more specific form. It is in no way intended to be exhaustive, but rather to include only the majority of the common laboratory procedures which the student will use, and to make available to him specific information concerning

iii

them. Many of these are, of course, amplified and extended in subsequent sections on the bacteriological diagnosis of the various infectious diseases. In the preparation of this material the writer has drawn heavily on the *Manual of Methods* of the Society of American Bacteriologists and on *Diagnostic Procedures and Reagents* of the American Public Health Association in an effort toward further standardization of laboratory procedure at this basic level. The chapter on streptococci has also been completely rewritten with closer integration of these bacteria as a group of pathogens, and a relating of the various clinical forms of infection on this common basis. The rapid advances of the past few years in bacterial genetics have necessitated a reconsideration of bacterial variation, and this chapter has likewise been entirely rewritten in a somewhat different form with consideration of the observed variations, the relation of these to one another, and finally the questions of underlying mechanisms. In conjunction with this, the earlier section on the effects of radiation has also been rewritten.

A considerable number of sections have also been rewritten. These include the sections on respiration and carbohydrate metabolism, in which greater emphasis has been placed on the cyclic nature of these processes together with integration of the modes of formation of the products of carbohydrate metabolism as a generalization of pyruvate metabolism. Sections on the biosynthesis of carbohydrate and amino acids have also been added, and the catalysis of these and the catabolic phases of metabolism related to nutritive requirements. Similarly, the discussion of the mechanisms of antibacterial action has been rewritten and extended, and related more closely to bacterial physiology. The section on hypersensitivity has been revised also, with correlation of clinical disease with basic immunological phenomena, and entirely new sections on Donovania, rickettsialpox and infectious hepatitis have been added.

Other more general changes include the addition of a considerable number of electron micrographs, and more recent data on the seasonal incidence of infectious disease. As to the former, only relatively recently has resolution been such that electron micrography has contributed to and become a valuable adjunct in the study of bacterial morphology. Its value is apparent in the demonstration of intracellular structures perhaps identical with nucleus-like chromatinic bodies, the convincing evidence of the origin and insertion of flagella, the elucidation of the details of L type reproduction, and other hitherto obscure problems. Electron micrographs have, therefore, been freely used to illustrate these and other matters, but have not been substituted for photomicrographs which remain, of course, invaluable to the student.

The writer is greatly indebted to his collaborators, Dr. F. B. Gordon who is responsible for the viruses, Dr. R. J. Porter who has continued his responsibility for medical parasitology, and Dr. J. W. Moulder who, with this edition, has taken over the general subject of bacterial physiology and has written the sections on respiration, carbohydrate metabolism and synthesis. These contributions are now recognized on the new title page coincident with the dropping of the name of Dr. E. O. Jordan as co-author. With the rewriting of this book through successive editions since Dr. Jordan's death thirteen years ago, his connection with it has become so tenuous that his name can no longer

be asked to carry the burden of responsibility for authorship, but is retained in the title as a token of the writer's deep affection and respect.

The number of colleagues who have taken the time and trouble to offer suggestions and criticisms is very great indeed. The writer's obligation to them all can hardly be overestimated, and he hopes that they will continue to offer such invaluable advice. Of these, the writer is especially indebted to Dr. Stuart Mudd and his co-workers for advice and assistance regarding electron micrographs and newer studies of bacterial morphology, to Dr. C. Robinow for photomicrographs of his chromatinic bodies, to Dr. Robinow and Miss Woutera van Iterson for electron micrographs of flagella, to Dr. R. W. G. Wyckoff for his superb micrographs showing phage generation, and to Dr. W. A. Jamieson who allowed the writer free access to the entire collection of electron micrographs of the Lilly Research Laboratories. Last, and by no means least, the writer is deeply appreciative of the help of Mrs. Gertrude Conklin and that of the staff of the W. B. Saunders Company in carrying this book through the press to its final form.

WILLIAM BURROWS

CONTENTS

THE HISTORY AND DEVELOPMENT
OF BACTERIOLOGY[1]

Bacteriology illustrates, perhaps better than any other branch of biology, the complex system of interrelationships among the sciences. Since the microorganisms are so small that they are invisible not only to the naked eye but to the eye aided by simple lenses, their very discovery necessarily depended upon the development of optical science to such a point that lenses of sufficient perfection and magnifying powers became available. It is not strange, therefore, that the many evidences of bacterial activity, such as the putrefaction and decay of organic matter, the infectious diseases of man and lower animals, the common fermentations and similar natural phenomena, should have been viewed in the past as semimetaphysical processes. Despite the entire lack of evidence, some of the older writers voiced a belief in the existence of such microorganisms. Fracastorius of Verona (1546) suggested a *contagium vivum* as a cause of disease, and von Plenciz (1762) accounted for the specificity of disease on the basis of microbic etiology. Although such speculations might be considered a result of prophetic insight, in all probability they were no more than guesses to which, in view of present knowledge, it is not difficult to ascribe significance. In the absence of the experimental approach, the fertility of philosophic speculation remains a doubtful quality.

Kircher had reported the observation of "minute worms" in the blood of plague patients in 1659, but it is doubtful that he ever saw the plague bacilli. The first concrete foundations of bacteriology were laid by a Dutchman, Antonj van Leeuwenhoek, in the latter part of the seventeenth and the early part of the eighteenth centuries. Leeuwenhoek, a man of indefatigable industry and great curiosity, held a political sinecure in his native town of Delft. In his odd time, of which he apparently had a good deal, he ground lenses and became the most skilful lens grinder of his time. He utilized these lenses in the construction of simple microscopes and spent many years of his long life (1632–1723) in examining a great variety of natural objects, with unremitting industry if without system, and in the course of his observations chanced to come across the organisms now known as bacteria. That he did in fact observe these creatures is evident from the drawings he left of them. In a communication to the Royal Society,[2] of which he was a member, he states,

[1] For details see Bulloch: *The History of Bacteriology*. Oxford University Press, New York. 1938.

[2] Phil. Trans. Roy. Soc., 1677, *11–12*:821. Observations, communicated to the publisher by Mr. Antony van Leeuwenhoek, in a Dutch letter of the 9th of Octob. 1676, Here English'd: Concerning little animals observed by him in rain- well- sea- and snow water, as also in water wherein pepper had lain infused.

"Having several times endeavoured to discover the cause of the pungency of pepper upon our tongue, and the rather, because it hath been found, that though pepper had layn a whole year in vinegar, yet it retained still its pungency; I did put about ⅓ of an ounce of whole pepper in water, placing it in my study, with this design, that the pepper thereby being rendered soft, I might be enabled the better to observe what I proposed to myself. This pepper having lain about three weeks in the water, to which I had added some snow water, the other water being in great part exhaled, I looked upon it the 24th of April, 1676 and discern'd in it, to my great wonder, an incredible number of little animals, of divers kinds; . . . The 4th sort of creatures, which moved through the three former sorts, were incredibly small and so small in my eye, that I judge, that if 100 of them lay one by another, they would not equal the length of a grain of coarse sand; and according to this estimate, ten hundred thousand of them could not equal the dimensions of a grain of such sand." Leeuwenhoek is known to have used only simple lenses, and the highest magnification he reached was approximately 300 diameters. Probably he did not observe objects as small as bacteria by transmitted light. He kept his method of illumination secret; it is not improbable that he used reflected light, similar to that used in present day ultramicroscopes. Although his observations did not lead to an immediate development of bacteriology, their significance was apparent to many of the scientific men of the day. Slare, in a comment on a report[3] on an epidemic among cattle, concludes with the remark, "I wish Mr. Leeuwenhoeck had been present at some of the dissections of these infected Animals, I am perswaded he would have discovered some strange Insect or other in them."

Nearly a century later, in 1786, the Danish zoologist, O. F. Müller, studied these microorganisms and succeeded in discovering many structural details of which his predecessors had been ignorant. He depicted several kinds of bacteria so accurately that they can be identified today as belonging to one or another of the chief divisions.

Another unequivocal advance was made by Ehrenberg (1795–1876). His principal work upon the "infusion animals" or "Infusionstierchen," as these organisms were then termed, was published in 1838 and brought together much more definite and detailed information concerning bacteria than had been previously secured. The chief merit of Ehrenberg's work lay in the system that it introduced into the study of microorganisms. He was able to establish a number of different groups among the organisms now known as bacteria, and recognized clearly the fundamental differences between the larger forms, such as the screw-shaped or spirally-twisted organisms, and certain of the true protozoa with which they had heretofore been classed. Some of the names which Ehrenberg conferred upon his "infusion animals," such as bacterium and spirillum, are still current in bacteriological nomenclature, although with changed signification.

[3] Phil. Trans. Roy. Soc., 1682, *13*:93. An abstract of a letter from Dr. Wincler, chief physitian of the Prince Palatine, Dat. Dec. 22. 1682 to Dr. Fred Slare, Fellow of the Royal Society, containing an account of a Murren in Switzerland, and the method of its cure. A further confirmation of the above mentioned Contagion, of its nature, and manner of spreading by way of Postscript from the ingenious Fred Slare, M. D. and F. R. S., Dat. March 27, 1683.

In the two or three decades succeeding Ehrenberg's work, considerable knowledge was amassed concerning the mode of development and physiology of bacteria, as well as their position in biological classification, but the labors of Dujardin, Perty, Cohn, Nàgeli and others, although important, are quite overshadowed by the work of Louis Pasteur (1822–1895).

Up to the period of Pasteur's investigations, the role played by bacteria in various familiar natural processes, such as putrefaction, decay and fermentation, had been, perhaps, vaguely suspected, but had not received conclusive demonstration. Pasteur, originally trained as a chemist, had done his early work on stereoisomerism. The formation of optically active amyl alcohol during the course of the lactic acid fermentation attracted him to the study of the

unquam sanguinem emittat. Nec tan
funt puri, quin, ubi eos per fpecult
tuerer, viderim crefcentem inter de
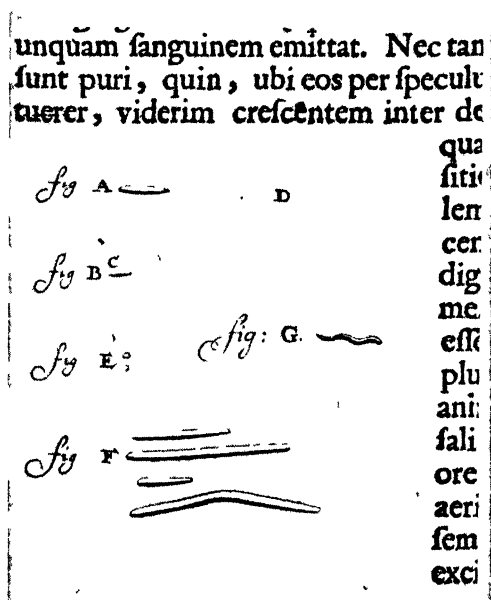
Fig. 1. The first pictorial representation of bacteria. Reproduced from *Arcana Naturae delecta ab Antonio van Leeuwenhoek*. Delphis Batavorum apud Henricum Crooneveld. 1695.

fermentation processes. The demonstration of the plant-like nature of yeast by Caignard-Latour and by Schwann, together with the asymmetric synthesis of amyl alcohol, led him to suspect that fermentation was a result of the activity of living cells. This view was strongly opposed by the chemists of the day, Liebig, Berzelius and Wöhler in particular, who regarded the presence of dead and dying yeast cells of importance only in that in the course of their own molecular disintegration they toppled over and dragged down certain complex organic molecules with which they were in contact. Pasteur's researches led him deeper into the morass of fundamental biology than, perhaps, he had anticipated, for, before the essentially biological basis of fermentation could be conclusively demonstrated, the problem of spontaneous generation of life from decomposing organic materials presented itself for solution.

For a great many years it had been generally held that living things, even organisms as large and complex as mice, were spontaneously generated during the course of the decomposition of organic substances. The experiments of the poet physician Redi (1626–1697), however, indicated definitely that maggots were not spontaneously formed from decomposing meat but were in fact fly larvae hatched from eggs deposited in the meat by flies. Spallanzani, an Italian monk, showed further that putrescible meat infusions, when properly heated, did not spoil and did not contain living organisms even though kept over long periods of time. Needham, an Irish priest, took issue with Spallanzani on the basis of similar experiments in which spoilage took place and living organisms appeared in spite of previous heating. A second series of elaborate experiments by Spallanzani corroborated his earlier findings and indicated the fallacies in Needham's experiments. Other experiments by Schulze, Schwann, Schröder and von Dusch indicated that no spontaneous generation took place but rather that the source of living organisms was the air in which they were suspended, a conclusion amply confirmed by the careful experiments of the Englishman Tyndall. The entire controversy was revived by the extended experimental work of Pouchet, which appeared to indicate a spontaneous generation of microorganisms in heated organic materials; and Pouchet created a great stir in French scientific circles—a stir in which Pasteur found himself.

The point at issue was, obviously, whether spoilage of such infusions took place as a result of the presence of microorganisms or whether the appearance of living organisms was a result of the decomposition—a general question of which the question of the biological basis of fermentation was a special case. Pasteur's extended and careful investigations showed beyond reasonable doubt that microorganisms were present in air, the numbers varying with the place from which the air came; that putrescible material which had been heated sufficiently to destroy all life would keep indefinitely with no evidences of either decomposition or the presence of living organisms; and, finally, that a common source of contamination of such sterilized material was air in which living microorganisms were suspended.

The fact that life came only from life (biogenesis) and was not spontaneously generated from non-living materials (abiogenesis) having been established,[4] it was not difficult to prove that fermentations resulted from the physiological activities of living, growing microorganisms. Furthermore, the specificity of fermentations, the fact that different kinds of fermentations were consequences of the activities of different kinds of microorganisms, grew out of this work. The transition from studies on fermentation to studies on infectious disease was not a difficult one to make, for there was a growing awareness among scientific men of the day of the similarities between the development of disease in an individual and the fermentation of sugar solutions.

Pasteur was led into a study of the "diseases" of beer and wine, processes which he found to be none other than secondary fermentations brought about through the activities of extraneous microorganisms, decompositions that resulted in the accumulation of end products of an undesirable nature. He

[4] Speaking scientifically. Philosophic speculation is beyond the scope of the present volume.

controlled the fermentative process by gentle heating followed by inoculation of the fermentable mixture with microorganisms which brought about the desired type of decomposition. The heating process has been termed "pasteurization" in his honor. A further development was a study undertaken at the request of the silk growers, of pébrine, a disease of silk worms which was assuming considerable economic importance. Here too a microbic etiology was discovered and practical control measures could be and were applied.

The so-called germ theory of disease had, at this time, permeated more and more generally into the scientific thought of the day, largely, perhaps, as a result of the strong support it received from Pasteur's researches on fermentation. Lister (1827–1912), an English surgeon, was one of the first to grasp the significance of Pasteur's work in relation to human disease, and put the new concepts into practical operation with his introduction of the antiseptic technique into surgical operations. The free use of strong carbolic acid brought about a marked decline in surgical mortality, which had been terrifically high in even the most minor operations. It remained, however, for Robert Koch (1843–1910), a German physician, to develop the experimental methods necessary to the proof of the causal relation between bacteria and infectious disease.

One of the greatest difficulties encountered by the early workers in bacteriology was that of separating bacteria from one another. That morphologically different types existed was clear, and, further, that organisms exhibiting the same morphology differed from one another in ability to produce disease had been apparent from the early work on pyemia. Perhaps one of the greatest single contributions to bacteriology is the method of isolation of bacteria in pure culture developed by Koch. It consisted, essentially, of culture on a semisolid medium, a nutrient environment solidified by the addition of gelatin or agar-agar—a method so simple and yet so effective that it is used practically unchanged today (p. 20). Koch had achieved what he had said only a year or two before was "impossible."

Although it was almost entirely through the works of Pasteur that bacteria and other microorganisms emerged from their relative obscurity as organisms of interest chiefly to the professional biologist and took a conspicuous position in natural science as a group of organisms whose activities and capabilities were full of a far-reaching significance for all mankind, the contributions of Koch and the German school were also of fundamental significance. If any one man can be looked upon as the founder of bacteriology, that man is Louis Pasteur. Likewise, the infant science owed its firm experimental foundation directly to Koch, a foundation without which it could never have become a science.

The isolation of bacteria in pure culture, coupled with the methods of staining developed by Koch, Ehrlich, Wiegert and others, so markedly stimulated the study of bacteriology that the ensuing decade or two, the eighties and nineties, became in truth the first golden age of that science. As the history of all science is, in essence, the history of its methodology, so in bacteriology discovery of the specific etiology of a variety of infectious diseases followed rapidly upon Koch's studies of anthrax and his isolation of the anthrax bacillus. Koch himself isolated and described the tubercle bacillus in 1882 and the cholera

vibrio the following year. Other discoveries tumbled in one after another with bewildering rapidity. Klebs described the diphtheria bacillus in 1883, and in 1884 Löffler isolated and studied this organism; Fraenkel discovered the pneumococcus in 1886, and the following year Weichselbaum isolated the meningococcus; Kitasato cultivated the tetanus bacillus in 1889, and in 1894 he and Yersin discovered the plague bacillus independently. These and many other similar discoveries made clear to the world in a striking fashion the significant implications of the new science.

Meanwhile, the investigations of the French workers had taken a somewhat different turn. Many years before, in 1796, Jenner, an English physician, observed that infection of human beings with cowpox, a disease of cattle closely resembling smallpox, protected against subsequent infection with smallpox. Pasteur, investigating first chicken cholera and later anthrax and swine erysipelas, succeeded in demonstrating a basic principle of immunology, that inoculation with attenuated microorganisms, those which by some sort of treatment had lost their virulence, resulted in the development of an increased resistance or immunity of the inoculated individual against later infection with the same organism. Pasteur's most striking application of this principle was in the development of a prophylactic treatment against rabies— an accomplishment with some elements of the dramatic because of the dread in which the disease was held. Not long afterward the American workers, Salmon and Theobald Smith, discovered that the inoculation of killed bacteria would also stimulate the development of the immune state. The cellular theory of immunity propounded by the Russian zoologist, Metchnikoff, was soon overshadowed by the discovery of tetanus antitoxin by von Behring and Kitasato and, shortly afterward, the discovery of diphtheria antitoxin by von Behring, and the precise studies of the humoral antibodies by Bordet and others; only in recent years has it come into its own. The utilization of the immune phenomena in the diagnosis of disease by Wassermann, Widal and others, Paul Ehrlich's development of his side-chain theory of immunity and his initiation of chemotherapy through the synthesis of salvarsan all contributed to the expansion and development of bacteriology. The discovery of the filterable viruses, apparently living agents too small to be seen with the most powerful microscopes, by Iwanowski, Beijerinck, and Löffler and Frosch brought up new problems, many of which are still unsolved today.

The new science, sired by Pasteur and nurtured by Koch, expanded beyond medicine and infectious disease into agriculture and the industrial fields. The genius of the Russian worker, Winogradsky, made possible the elucidation of the perplexing problems of soil fertility by his isolation of autotrophic bacteria which oxidized ammonia to nitrites and nitrates. The discovery of the nitrogen-fixing bacteria living symbiotically with leguminous plants by Hellriegel and Wilfarth and of the free-living nitrogen-fixing forms by Winogradsky and by Beijerinck further clarified the puzzles of soil fertility and indicated the important functions of the soil bacteria. The discovery of the bacterial etiology of pear blight by Burrill and the isolation of bacteria responsible for many other diseases of plants have shed new light on old agricultural problems. It has also been discovered that other kinds of bacteria impart the characteristic flavors or aromas to butter, cheese and other dairy products; and that still

others determine the success or failure of various industrial processes, such as the retting of flax, the tanning of hides and, perhaps, the curing of tobacco.

Thus, in the space of only a few decades, bacteriology had become a full-blown, vigorous young science, fully capable of standing on its own feet—and this from Leeuwenhoek's "little animals" that behaved in such curious ways.

The outstanding success of the practical applications of bacteriology should not be allowed to overshadow the fact that it owes its present important place among the biological sciences quite as much to its general scientific significance. It has been often pointed out that bacteriology has produced a change in man's conceptions of the world around him so sweeping as almost to deserve the term "revolutionary." Up to the middle of the nineteenth century the character of many of the most familiar natural processes, such as decay, fermentation and the like, was entirely misunderstood; contemporary spontaneous generation of at least the lower forms of life was the generally accepted belief of most scientific men; infectious diseases were not sharply differentiated from one another and the most fantastic hypotheses were advanced to explain their existence. Although the great mass of material phenomena elsewhere had been brought into apparent orderliness and system, here was a region in which the unscientific imagination rioted in mystery and extravagance. The penetration of this realm of obscurity by the discoveries of bacteriology gave the human race for the first time in its history a rational theory of disease, dispelled the myths of spontaneous generation, and set the process of decay and kindred phenomena in their true relation to the great cycle of living and non-living matter. The new conception of the microscopical underworld which bacteriology brought into biological science must be reckoned as a conspicuous landmark, and, in so far as it has changed the attitude of man toward the universe, should be regarded as one of the most important triumphs of natural science.

Underlying all the applications of bacteriology are certain fundamental facts and principles concerning the structure, mode of development, and general physiologic requirements and capabilities of bacteria themselves. This subject matter constitutes the ground work of bacteriology, and is essential not only to a proper comprehension of the present practical applications of bacteriology, but also to the further development of the science.

Chapter 2

LABORATORY METHODS FOR THE
STUDY OF BACTERIA[1]

The study of bacteria in the laboratory by the various procedures of isolation in pure culture, cultivation, microscopic examination, and characterization by biochemical and immunological methods necessarily begins before detailed consideration of their nature and properties can be completed. The basic laboratory procedures are summarized here in didactic form, and their rationale will become apparent later.

STERILIZATION

The determination of the character and properties of a given species of bacteria is necessarily based on their study in pure culture, *i.e.*, their separation from other microorganisms. Since bacteria and other microorganisms such as fungi are ubiquitous, all material coming in direct contact with the bacteria under study must be subject to preliminary sterilization. The sterilizing agent most commonly used is heat, dry or moist, but occasionally other methods, such as filtration of liquids through bacteria-proof filters, are desirable.

Glassware and Instruments. The usual glassware includes flasks, petri dishes, test tubes and pipettes which must, of course, be scrupulously clean. Flasks and tubes are plugged with nonabsorbent cotton which prevents entry of bacteria after sterilization, and a cotton plug is inserted in the mouth end of the pipettes. Petri dishes and pipettes may be placed in cans with covers or wrapped in paper to maintain sterility. Surgical instruments are wrapped in paper or towels, syringes separated and wrapped in paper, hypodermic needles placed in plugged test tubes, and other equipment similarly prepared.

Sterilization is effected in a hot air oven, electric or gas fired. The material to be sterilized is placed in the oven without crowding and the temperature raised to 170° to 180° C. and maintained for a period of not less than two hours. If appropriate temperature control equipment is not available, a slight browning of the cotton plugs is taken as indicative of sterilization, but this is a doubtful procedure.

In some instances sterilization by heating to dull red in a bunsen flame is exceedingly useful as in the flaming of forceps tips, the platinum or nichrome wire needles and loops used for transferring bacteria, and the lips of test tubes before and after transfer of bacterial culture.

Surgical instruments, hypodermic needles and syringes, and similar equipment may be sterilized with respect to the vegetative forms of bacteria by boiling in water or 1 per cent bicarbonate solution for 3 to 5 minutes, but this does not suffice to destroy the spores of bacteria and fungi.

Culture Media and Other Liquids. Material containing water cannot, of course, be sterilized by dry heat, but moist heat is a more effective sterilizing agent in any case.

[1] For the most part the methods described here are identical with or closely follow those of the *Manual of Methods for the Pure Culture Study of Bacteria* of the Society of American Bacteriologists and *Diagnostic Procedures and Reagents*, A.P.H.A., 2nd ed., 1945.

Intermittent Sterilization. As indicated above, boiling water does not provide a high enough temperature to bring about complete sterilization. Certain kinds of liquid culture media, however, are affected unfavorably by moist heat at higher temperatures, and these may be sterilized by intermittent sterilization. This is based on the assumption that the vegetative cells of microorganisms are destroyed at 100° C. in the presence of water, and that the surviving spores will germinate and these vegetative cells will be destroyed in turn by exposure to 100° C. In practice the material to be so sterilized is exposed to free-flowing steam in an Arnold sterilizer for 30 minutes, removed and incubated until the next day and the steaming repeated, and a third incubation and steaming is included as a safety factor so to speak. The method is not too successful in that spores not infrequently show a delayed germination, and spores of obligate anaerobic bacteria may not germinate. Sterilization by the intermittent method is, however, seldom required.

Autoclave Sterilization. The most efficient method of sterilization is that effected by steam at temperatures above 100° C. which may be produced when the steam is under pressure. The devices employed in the use of steam under pressure include the common pressure cooker, autoclaves of various designs and dressing sterilizers, which are autoclaves to which vacuum can be applied in order to dry fabrics that have been sterilized. Steam is generated directly in the apparatus or supplied by connection to a high pressure steam line.

As in the case of dry heat sterilization, the material to be sterilized must not be packed too tightly in the autoclave. The temperature is raised to 120° C. and maintained for a period of time depending on the material to be sterilized. As short a time as 15 minutes suffices for small volumes of material, as in culture tubes, loosely spaced, but if the tubes are packed in baskets 20 minutes or possibly more should be allowed. For larger volumes, as in flasks, longer periods of exposure are necessary, *e.g.,* for 500 ml. quantities 30 to 40 minutes. The period of time is that elapsing after the temperature is reached and until the pressure is shut off; the preliminary heating as pressure is built up and the slow fall in pressure at the end are not included. No attempt should ever be made to reduce the pressure rapidly after sterilization, for liquids will boil out of their containers.

Sterilization by Filtration. It is often desirable to sterilize solutions such as bacterial cultures in liquid media, solutions of substances relatively unstable to heat such as certain sugars and the like, without subjecting them to heat at sterilizing temperatures. These may be sterilized by filtration through filters of such fine porosity that bacteria are held back. The method of sterilization by filtration is particularly useful in obtaining soluble bacterial products such as toxins.

Several kinds of bacteria-proof filters are available. The Berkefeld filters are made of infusorial earth in three porosities, V (*viel*) or coarse, N (*normal*) and W (*wenig*); of these the V filters remove most but not all bacteria, the N filters usually sterilize, and the W filters are used for very small microorganisms. The Chamberland filter, of unglazed porcelain, is made in graded porosities, L1, L2, L3, etc., and of these the L3 is roughly equivalent to the Berkefeld N. The Seitz or Seitz-Werke filter is made of metal and the filtering element is an asbestos pad which is the equivalent of the Berkefeld N filter. Sintered glass filters are available in a variety of porosities which in the pyrex filters are designated C (coarse), M (medium), F (fine) and UF (ultrafine). The UF filter is bacteria-proof while the others are not.

Filtration is accomplished by a pressure differential which may be obtained by positive pressure on the liquid to be filtered, or negative pressure on the filtrate, and usually a differential of 20 to 30 cm. Hg suffices for reasonably rapid filtration without foaming. The Berkefeld and Chamberland types are prone to develop leaks, either as cracks in the filter candle, or where the candle is cemented to the metal portions, and the integrity of the filter must be controlled frequently by including a trace organism such as *Bact. prodigiosum* in the liquid to be filtered and by culture of the filtrate. Whatever filter is used it must, of course, have been sterilized, usually in the autoclave, prior to use.

PREPARATION OF CULTURE MEDIA

The nutritive requirements of bacteria vary greatly; some will grow readily in the so-called synthetic media containing inorganic salts, including an am-

monium salt, together with a simple organic compound such as glucose or asparagin as a source of carbon and energy. In general, bacteriological media have been developed on a trial and error basis about a basic nutrient medium containing peptone and the water-soluble material, largely extractives, of muscle tissue. The source of the latter may be commercial preparations of meat extract but a somewhat better medium is obtained if these substances are extracted from fresh meat, beef or veal for the most part.

The basal nutrient solution may be modified in a variety of ways. Thus, it may be solidified by the addition of gelatin or agar, or enriched with serum, ascitic fluid, defibrinated blood and the like to support the growth of the more fastidious bacteria. Various sugars may be added, together with an acid-base indicator, for the determination of the fermentative properties of bacteria; nitrate or tryptophane may be added to test for ability to reduce nitrate to nitrite, form indol from tryptophane, etc. Or culture media may be prepared which are differential and accentuate physiological differences, and specific inhibitory agents may be added to give selective media which allow the growth of some kinds of bacteria and suppress that of others. These various characteristics may be combined, of course, as in the case of an enriched medium which is both selective and inhibitory. In consequence, there is a great variety of bacteriological culture media, but they are in large part interrelated modifications of a basal nutrient solution. The constituents and method of preparation of a few of the commonly used culture media may be reviewed here.

Basal Media

Meat Extract Broth and Agar. The medium usually has the following composition:

meat extract	3 gm.
peptone	5 gm.
(NaCl	5 gm.)
(agar	15 gm.)
distilled water	1000 ml.

It is necessary to include the sodium chloride if the medium is to be used for serological work or enriched with blood, and the agar is omitted if a liquid medium is desired. The ingredients are dissolved in water. Agar has the property of not dissolving or of its gels not melting unless it is heated to boiling, but its solution does not gel until the temperature reaches about 40° C. If agar is used it is necessary to boil the solution or heat it in the autoclave, and clarify by filtration. The initial solution is usually slightly acid and the reaction is adjusted to *p*H 7 by the addition of alkali.

Meat Infusion Broth and Agar. The infusion media differ from the extract media in that the extractives are obtained by infusion of fresh meat, but otherwise the composition is the same, *viz.*:

extract of 400 to 600 gm. fresh lean beef or veal	
peptone	5 gm.
(NaCl ..:....................................	5 gm.)
(agar	15 gm.)
distilled water to	1000 ml.

The meat is ground in a meat chopper, suspended in 1 liter of distilled water, and infused overnight in the refrigerator. The following morning the fat is skimmed off with absorbent cotton and the infusion squeezed through muslin and made up to 1000 ml. The other ingredients are added, the reaction adjusted to *p*H 7, the solution heated to 100° C. for about 20 minutes, regardless of whether agar is used, filtered through coarse paper and made up to 1000 ml. The heating is for the purpose of coagulating the tissue proteins dissolved during infusion so that they can be removed by filtration.

Media for Biochemical Tests

Sugar Broths. The sugar broths are meat extract or meat infusion broth to which the required carbohydrate has been added to a concentration of 1 per cent. Unless the carbohydrate is stable to autoclave sterilization, it must be sterilized separately by filtration in concentrated solution and added aseptically to the sterile broth. It is customary to add an acid-base indicator dye, bromthymol blue or bromcresol purple, and the medium may be dispensed in culture tubes containing an inverted vial that fills during sterilization and serves to collect gas evolved in the fermentation of the sugar.

Nitrate Broth. To test for ability to reduce nitrate, bacteria are cultured in nitrate broth, infusion or extract broth containing 0.1 per cent KNO_3. The culture is tested for the presence of nitrite with sulfanilic acid and a-naphthylamine reagents.

The sulfanilic acid reagent is prepared by dissolving 8 gm. of sulfanilic acid in 1000 ml. of 5N acetic acid. The a-naphthylamine reagent (a-amidonaphthalene acetate) is prepared by dissolving 5 gm. of a-naphthylamine in 1000 ml. of 5N acetic acid, and clearing by filtration through absorbent cotton.

To test for nitrite add 2 ml. of each of the two reagents to 3 to 5 ml. of culture; the development of a rose color, the nitroso reaction, indicates the presence of nitrite.

Tryptophane Broth. The test for the production of indol from tryptophane can often be carried out with cultures in meat broth or peptone water, the latter containing peptone but no meat extract or infusion. The important factor is an adequate amount of tryptophane in the peptone. Some peptones are deficient, but a peptone marketed under the name tryptone contains adequate amounts of the amino acid.

The presence of indol is tested for after 2 to 4 days' incubation of the culture by layering a small amount of Ehrlich's reagent on the surface of the culture; the development of a red color at the interface indicates the presence of indol. If a color does not appear within 1 minute, add a small amount, equal to that of the Ehrlich reagent, of saturated aqueous solution of potassium persulfate.

Ehrlich's reagent consists of 4 gm. of *p*-dimethylaminobenzaldehyde dissolved in a mixture of 380 ml. of ethyl alcohol and 80 ml. of concentrated hydrochloric acid.

Lead Acetate Agar. This medium is meat extract or meat infusion agar containing 0.05 per cent basic lead acetate and dispensed in culture tubes without slanting. It is usually prepared by mixing equal amounts of sterile double strength basal medium, *i.e.*, containing double amounts of the ingredients, and sterile 0.1 per cent aqueous solution of basic lead acetate.

The medium is inoculated by stab and the production of hydrogen sulfide from the sulfur-containing amino acids of the peptone is indicated as a blackening or browning of the medium.

Meat Extract and Meat Infusion Gelatin. These media are the basal media solidified by the addition of 12 per cent gelatin and are dispensed in culture tubes without slanting, and inoculated by stab. They indicate whether or not gelatin is liquefied by the microorganism under study. Gelatin liquefies at 37° C. and gelatin cultures should be incubated at 22° C. In the case of those bacteria which will not grow at this lower temperature, the culture may be incubated in the usual way and after incubation tested for ability to solidify by chilling. Gelatin liquefaction sometimes occurs slowly and cultures should be retained for not less than two weeks unless positive earlier.

Milk. Milk is an adequate culture medium for many bacteria without modification other than the addition of an indicator, bromcresol purple or litmus in an amount of 5 ml. of a 0.25 per cent alcoholic solution per liter. Fresh skim milk may be used, but skim milk powder is usually more convenient. One liter of medium is made from 150 gm. of milk powder, first rubbed up in a mortar and then diluted to volume. Medium prepared from powdered milk may be autoclaved, but that from fresh milk is best sterilized by the intermittent method.

Because of its protein and carbohydrate content milk cultures undergo a variety of biochemical changes, *viz.*:

(1) The development of an alkaline reaction, usually after 3 to 4 days' incubation.

(2) The development of an alkaline reaction with precipitation of the casein as a rennet curd, with or without reduction of the indicator.

(3) The development of alkalinity and rennet curd precipitation, followed by peptonization or digestion of the curd, resulting in a clearing and brownish discoloration; the indicator is usually reduced by the time digestion is apparent.

(4) Acid formation, usually in 24 hours, with or without reduction of the indicator.

(5) Acid formation and precipitation of the casein, usually with reduction of the indicator.

Enriched Media. Basal infusion media may be made richer by the use of special peptones, such as neopeptone and proteose peptone, and the addition of small amounts of glucose and phosphate buffer. In addition, fluids such as serum, ascitic fluid or defibrinated blood may be added.

Blood Agar. The most common and most useful enriched medium is blood agar—veal or beef infusion base to which defibrinated whole blood is added. The blood is usually taken from the rabbit, sheep or horse under sterile conditions and defibrinated as it is drawn by shaking with glass beads. The sterile infusion agar base is liquefied by heating, cooled to about 45° C., the sterile blood added to 10 per cent concentration, and the medium dispensed before it solidifies, usually in petri dishes or as slants in culture tubes. The entire operation is carried out under sterile conditions and the medium incubated for 24 hours to detect contamination, and then stored in the refrigerator. This medium is rich enough to support the growth of almost all the fastidious pathogenic bacteria and has the advantage that hemolysis is shown directly in the cultures.

Cystine Blood Agar. This is simply blood agar medium further enriched by the addition of 0.01 per cent cystine and 1 per cent glucose to the basal medium prior to the addition of blood as above. It is useful primarily in the cultivation of *Pasteurella tularensis.*

Chocolate Agar. This medium is heated blood agar and is particularly useful for the cultivation of the gonococcus and meningococcus. Formulae vary somewhat, some workers preferring a beef heart infusion (see below) instead of muscle tissue infusion and proteose peptone instead of peptone. The basal infusion medium is enriched by the addition of defibrinated blood as in the preparation of blood agar, but after the addition of the blood, it is heated slowly in a water bath until the medium has a chocolate brown color; if the heating is excessive the blood coagulates and the finished medium will contain clumps of cooked blood, so that the temperature should not rise above 75° C. at the most.

Other Infusions. The use of infusions of tissues other than beef or veal muscle is sometimes desirable for the cultivation of certain bacteria. Beef liver infusion is prepared in the same manner as that described for the other infusions and, for culture of Brucella for which liver infusion medium is particularly useful, the basal medium is enriched by the addition of 1 per cent egg albumin. Beef heart infusion is also prepared in the same manner as beef muscle infusion and the basal medium completed by the addition of a peptone, sodium chloride, etc.

Löffler's Medium. This medium is used primarily for culture of the diphtheria bacillus and consists of 3 parts of sterile beef serum and 1 part of meat infusion basal medium containing 1 per cent glucose. The serum is added to the sterile base, mixed, dispensed in culture tubes, slanted and sterilized by the intermittent method. Sterilization may be carried out on three successive days in the autoclave at 15 pounds steam pressure without letting the air out, or it may be held at 15 pounds for 15 minutes and the air allowed to escape slowly while maintaining pressure and, when all the air has escaped, holding for an additional 15 minutes, and after sterilization allowing the pressure to fall very slowly. The medium is solid because of coagulation of the serum and sterilization must be carried out carefully to avoid the formation of bubbles.

Blood Culture Medium (Kracke). This is a highly enriched medium containing beef heart infusion and brain suspension and is used primarily for blood culture of pathogenic bacteria in infections accompanied by bacteremia. Its composition is:

heart infusion	750 ml.
brain suspension	250 ml.
sodium citrate	1 gm.
dextrose	10 gm.
peptone	10 gm.
dibasic sodium phosphate	2 gm.
sodium chloride	4 gm.

The heart infusion is prepared in the same manner as muscle tissue infusion described earlier. The brain suspension is prepared by maceration of 500 gm. of fresh brain in water, straining through a metal strainer and heating slowly to boiling with constant stirring. The heating coagulates the brain tissue and leaves it in a state of fine suspension; it must not be filtered. The other ingredients are added to the mixture of heart infusion and brain suspension, the *p*H is adjusted to 7.4, the mixture is dispensed in 50 ml. amounts in 100 ml. flasks (to leave room for an inoculum of 10 to 20 ml. of patient's blood) and sterilized by autoclaving. Cultures in this medium are essentially enrichment cultures and should be subcultured on appropriate agar media such as blood agar for streptococci and staphylococci, liver infusion agar for Brucella, etc.

Dorset's Egg Medium. This medium is used for the cultivation of the tubercle bacillus and consists of 4 fresh eggs and 25 ml. of 0.85 per cent sodium chloride solution. The eggs are scrubbed with a brush in soap and water, rinsed, dried, put into a wire basket and dipped into 95 per cent alcohol, drained and the remaining alcohol ignited. They are then broken using aseptic technique and the whites and yolks placed in a sterile container. Twenty-five ml. of sterile salt solution is added, the whole mixed well with a sterile egg beater, dispensed in culture tubes and slanted, and sterilized by the intermittent method described for Löffler's medium.

Differential and Selective Media. The various differential and selective media have been designed to facilitate the isolation of one kind of bacteria in the presence of many other kinds, by accentuating physiological differences and/or inhibiting the growth of the unwanted forms. Of the pathogenic bacteria, those most commonly encountered admixed with large numbers of closely related forms are the enteric bacilli, and many of the selective media are selective for these bacteria. The simplest of the purely differential media consist of meat extract or infusion agar base together with a sugar and an indicator. The most satisfactory indicator in most cases is bromthymol blue, and the colonies of the sugar-fermenting bacteria are yellow while those of the non-fermenters are blue. Of the selective agents bacteriostatic dyes are often used, and others include bile, tellurite, etc.

Endo's Medium. This medium consists of meat extract agar to which has been added lactose and Schiff's reagent, basic fuchsin decolorized with sulfite. When the lactose is fermented aldehyde intermediates restore the color to the fuchsin and colonies of lactose-fermenting bacteria are red while those of the non-lactose-fermenters are white. The composition of the medium is:

hot melted extract agar	1000 ml.
sodium carbonate, 10% aqueous	10 ml.
lactose	10 gm.
sodium bisulfite, 10% aqueous	10 ml.
basic fuchsin, 3% alcoholic	10 ml.

The *p*H of the medium is raised by the carbonate and should be 7.6 to 8.0.

Eosin-Methylene Blue Agar. This medium is sterile meat extract agar to which is added 5 ml. of a 10 per cent solution of lactose, 2 ml. of 2 per cent aqueous eosin and 2 ml. of 0.5 per cent aqueous methylene blue. These solutions are added aseptically to the melted and cooled medium and the mixture is dispensed in sterile petri dishes.

Desoxycholate-Citrate Medium. This medium is differential in that it contains lactose and neutral red as an indicator, and selective in that it contains bile salt, and is one of the

most satisfactory and widely used media for the isolation of Salmonella and dysentery bacilli. It contains:

meat infusion	1000 ml.
peptone	10 gm.
agar	20 gm.
lactose	10 gm.
sodium citrate	20.6 gm.
sodium desoxycholate	5 gm.
lead chloride, 0.35% aqueous	1 ml.
ferric ammonium citrate	2 gm.
neutral red, 1% aqueous	2 ml.

The complete medium is not stable on storage. The peptone and meat infusion are mixed, the pH adjusted to 7.5. The solution is boiled for 3 minutes, lost water added, and filtered through paper. The agar is added to the hot solution together with 5 ml. of N NaOH and it is allowed to stand for 15 minutes, then steamed at 100° C. for 20 minutes. Then the lactose, citrate, desoxycholate and lead chloride are added. This may be stored and remelted for use. Just before use, the agar is heated to 100° C., the ferric ammonium citrate added and the pH adjusted to 7.4 using phenol red as an indicator, and finally the neutral red is added and the medium dispensed in plates. Note that it is not autoclaved.

Bismuth Sulfite Medium (Hajna). This medium is used primarily for the isolation of the typhoid bacillus from fecal specimens and is highly specific for that organism. As originally developed it was difficult to prepare consistently but Hajna's modification is highly satisfactory. It consists of a meat extract agar base containing 0.5 per cent glucose, the bismuth sulfite mixture and brilliant green. The bismuth sulfite mixture is prepared as follows:

(1) Dissolve 80 gm. of anhydrous bismuth sulfite in 400 ml. of hot water with stirring.

(2) Make a paste of 24 gm. of bismuth citrate in 40 ml. of water, add 12 ml. of concentrated ammonia and stir until a sol is formed. Dilute to 200 ml. with distilled water and mix to give a solution.

(3) Mix (1) and (2) and add 42 gm. of anhydrous diabasic sodium phosphate and mix until dissolved.

(4) Dissolve 4 gm. of ferrous sulfate in 50 ml. of distilled water containing 2 drops of concentrated hydrochloric acid, and add 40 ml. of this solution to mixture (3). Mix and boil gently for about 2 minutes until slate gray in color. This is the bismuth sulfite mixture and it is stable for about 2 months.

For use add 70 ml. of the bismuth sulfite mixture and 4 ml. of a 1 per cent aqueous solution of brilliant green to each 1000 ml. of the hot melted agar base and autoclave for not more than 10 minutes, and dispense in petri dishes. The complete medium may be kept for perhaps 2 weeks in the refrigerator without autoclaving, and should be autoclaved just prior to dispensing.

Tetrathionate Enrichment Broth. Nutritive enrichment broths are used for preliminary culture of specimens followed by subculture on selective differential media. Tetrathionate broth and selenite F broth (see below) are very useful in the isolation of Salmonella from fecal specimens. The active agents in tetrathionate broth are bile salts, brilliant green and tetrathionate, the last formed by oxidation of thiosulfate with iodine just prior to inocula-tion. Its composition is as follows:

proteose peptone		5 gm.
bile salts (Bacto)		1 gm.
distilled water		1000 ml.
calcium carbonate		10 gm.
sodium thiosulfate		30 gm.
brilliant green, 1% aqueous		11 ml.
iodine, 25% aqueous	2.5 ml. per	100 ml.
⌠ iodine	25 gm.	
⎨ potassium iodide	20 gm.	
⌡ water to	100 ml.	

The peptone and bile salts are dissolved in water and calcium carbonate added, and autoclaved. The sodium thiosulfate and brilliant green are added to the sterilized medium and it is dispensed in tubes or small flasks. Just prior to inoculation add the iodine solution in the proportion of 2.5 ml. per 100 ml.

Selenite F Broth. This medium contains selenite as an inhibitory agent and its composition is:

sodium hydrogen selenite	4 gm.
peptone	5 gm.
lactose	4 gm.
sodium phosphates (anhydrous)	10 gm.
distilled water	1000 ml.

It is necessary to determine the proportions of acid and basic phosphates which will give a final reaction of *p*H 7.0 to 7.1 with the peptone and the lot or brand of selenite used. The ingredients are dissolved in warm water, and then brought to a boil and dispensed. The medium is not autoclaved.

Chocolate-Tellurite Medium. This is a chocolate agar prepared by adding 10 per cent of defibrinated blood to meat infusion base agar as described earlier, but modified by the addition of 150 ml. of sterile 0.3 per cent aqueous solution of potassium tellurite to every 1000 ml. of infusion base. It is used for culture of diphtheria bacilli.

Petragnani's Medium. This is a medium used for culture of tubercle bacilli and contains malachite green to inhibit the growth of gram-positive bacteria and molds. Its composition is:

milk	900 ml.
potato flour	36 gm.
peptone	6 gm.
pieces of potato, size of an egg	6
eggs	24
egg yolks	6
glycerin	72 ml.
malachite green, 2% aqueous	60 ml.

The potatoes are cut into thin slices, the milk, potato flour and peptone added, and the whole cooked in a double boiler for 2 hours with stirring. The eggs and egg yolks are broken together, the glycerin and malachite green added, and the mixture shaken well. The milk-potato mixture is cooled to 50° C., the egg-glycerin-dye mixture added and mixed well, and the whole is filtered through gauze, dispensed into tubes, slanted and sterilized by the intermittent method described for Löffler's medium.

MICROSCOPIC EXAMINATION OF BACTERIA

The direct observation of bacteria is an essential part of their study. Characteristics such as the shape and grouping of the cells, the presence or absence of structures such as capsules, flagella and spores, the reaction to differential stains, and the like are of considerable differential significance.

Motility. Some bacteria are motile by virtue of organs of locomotion, flagella, which are long, sinuous appendages attached to the cell. Motility can be observed only in the living state, of course, and the living bacteria are mounted in a drop of liquid on a cover slip inverted over a hollow ground or depression slide. Motility is to be differentiated from brownian movement; the latter is a dancing irregular movement, while motile bacteria move across and in and out of the microscopic field, and the movement may be surprisingly rapid.

For demonstration of motility the culture should be young, not more than 18 hours old. Bacteria from cultures on agar media must be suspended in saline, while broth cultures may be used directly. A drop of suspension or culture is placed in the center of a cover slip. The depression in the hollow ground slide is lined with petrolatum on the surface around the depression, and inverted over the cover slip. The cover slip sticks to the slide and evaporation is prevented by the seal. The preparation is turned over and examined under the oil immersion lens. Unstained bacteria are difficult to see and the diaphragm should be closed to a small aperture; it is best to focus first on the edge of the drop.

Staining. Bacteria may be stained or dyed with aniline dyes, more readily with the basic dyes.[2] Staining may be with a single dye, or simple stain, most commonly crystal violet, methylene blue or basic fuchsin, with mixed dyes or polychrome stains, or by differential methods based on the relative affinity of different bacteria or different structures of the bacterial cell for the stains used. The nature of the staining process has been a matter of considerable controversy, in large part as to whether it is fundamentally a physical or chemical process. It is now generally agreed that it is chemical in nature and the mechanism an ionic interchange between the basic dye and the acidic portions of the protoplasm—nucleic acids and their compounds—with the formation of insoluble dye-nucleotide compounds that do not diffuse out of the cell.

Stain Solutions. The composition of stain solutions varies considerably in the literature. The several, frequently somewhat indefinite, formulae have been interpreted by the Committee on Biological Stains and are given here in the emended form recommended by that Committee. In older formulae the dye content is often given as ml. of a saturated alcoholic solution, and the accompanying table gives the solubilities of those most commonly used, in water and in 95 per cent alcohol.

SOLUBILITIES OF COMMON BACTERIOLOGICAL STAINS[2]

Color Index Number	Name	Per Cent Soluble in	
		Water	95% Alcohol
655	Auramine O	0.74	4.49
681	Crystal violet (chloride)	1.68	13.87
676	Fuchsin, basic (pararosanilin hydrochloride)	0.26	5.93
657	Malachite green (oxalate)	7.60	7.52
922	Methylene blue (hydrochloride)	3.55	1.48
841	Safranin	5.45	3.41
925	Toluidine blue O	3.82	0.57

Löffler's Alkaline Methylene Blue:
   Solution A:
      methylene blue (90% dye content) . . . .   0.3 gm.
      ethyl alcohol (95%)   30   ml.
   Solution B:
      dilute KOH (0.01% by weight)   100   ml.
The two solutions are mixed in the above quantities.

Ziehl's Carbol-Fuchsin:
   Solution A:
      basic fuchsin (90% dye content) . . . .   0.3 gm.
      ethyl alcohol (95%)   10   ml.

[2] See *Biological Stains.* Prepared by the Commission on the Standardization of Biological Stains, H. J. Conn, chairman. 3rd ed. Geneva, N. Y. 1940.

Solution B:
        phenol .... 5 gm.
        distilled water .... 95 ml.
Mix the two solutions in the above quantities.

Ammonium Oxalate Crystal Violet (Hucker):
    Solution A:
        crystal violet (90% dye content) .... 2 gm.
        ethyl alcohol (95%) .... 20 ml.

    Solution B:
        ammonium oxalate .... 0.8 gm.
        distilled water .... 80 ml.
Mix the two solutions in the above quantities.

Safranin:
        safranin (2.5% solution in 95% alcohol) .... 10 ml.
        distilled water .... 100 ml.

Albert's Diphtheria Stain (Laybourn):
        toluidine blue .... 0.15 gm.
        malachite green .... 0.02 gm.
        acetic acid (glacial) .... 1 ml.
        ethyl alcohol (95%) .... 2 ml.
        distilled water .... 100 ml.

Preparation of Smears. Slides should be clean and free from all grease, and may be cleaned in soap and water, thoroughly rinsed, and stored in alcohol or xylol. Before use a slide is passed through the bunsen burner flame two or three times. A drop of distilled water is placed on the cooled slide and a small amount of bacterial growth suspended in it, spread and allowed to dry in air. The film is then fixed by passing through the burner flame two or three times, or, for some stains, by immersion in absolute methyl alcohol, glacial acetic acid or other fixatives.

Procedure of the Simple Stain. The fixed smear is covered with stain, allowed to stand for 30 to 60 seconds, rinsed off with tap water, excess water removed by blotting and allowed to dry in air. Bacteria are usually examined under the oil immersion objective, and the oil can be placed directly on top of the smear.

The Gram Stain. Of the differential stains, the Gram stain is one of the most valuable and most generally applied. The procedure is essentially one of staining with crystal violet, mordanting with iodine solution, decolorizing with alcohol and counterstaining with a dye of contrasting color. The stain was originally developed by the histologist Gram in an effort to stain bacteria in tissues differentially, and has been subject to many modifications. By this procedure bacteria are separated into two groups, those which retain the crystal violet and are said to be *gram-positive,* and those which are decolorized and stain with the counterstain which are *gram-negative.* The distinction is not always sharp for there is considerable variation in ease of decolorization, some gram-positive bacteria such as the pneumococcus become gram-negative after they die, and some bacteria are gram-variable. This last group is not large enough to detract from the practical value of the stain.

The Mechanism of the Gram Stain. Both the crystal violet and the iodine are highly specific while the alcohol and the counterstain are not. Almost all other dyes, even methyl green which differs by only one methyl group from crystal violet, are unsuitable, either being retained or removed by alcohol whether or not the iodine is applied. A few other reagents, notably

HgCl$_2$, may be substituted for iodine, and stannous chloride, pyrogallol and freshly prepared solutions of hydroquinone show some slight activity.

However arbitrary Gram's method of staining may appear, the reaction is apparently associated with fundamental differences between the gram-positive and gram-negative organisms. There is, for example, a pronounced correlation between it and resistance to the bacteriostatic action of certain dyes and antibiotic substances, and to the action of other chemical and physical agents. The nature of the gram reaction has, therefore, been of considerable interest and three general types of theories to explain the differential staining have been developed:

(1) The theory of differential membrane permeability was proposed by Benians,[3] whose experimental evidence was consistent with the view that while the cell membrane of both gram-positive and gram-negative bacteria is permeable to the crystal violet and iodine reagents, that of the former is not permeable to the alcohol-soluble dye-iodine complex and hence the color is retained.

(2) The colloidal chemical theory was advanced by Stearn and Stearn[4] who postulated a relationship between the isoelectric point of the cell protoplasm and its affinity for basic dye on the basis of the negative response of gram-positive bacteria at acid pH's, and related evidence; the isoelectric point of gram-positive bacteria is at a lower pH than that of the gram-negative bacteria and therefore the former have a stronger affinity for the basic dye.

(3) A morphological theory was developed principally by Churchman.[5] His evidence strongly supported the view that the protoplasm of all bacteria is gram-negative and that the gram-positive forms are covered by a sheath or envelope of gram-positive material, *i.e.*, the gram-positive cell consists of a gram-negative "medulla" and a gram-positive "cortex."

More recent work has substantiated this third hypothesis. Henry and Stacy[6] were able to remove an outer layer from gram-positive bacteria by treatment with bile to leave a gram-negative cell or "cytoskeleton." The bile extract, not in itself gram-positive, contained protein, polysaccharide and the magnesium salt of ribonucleic acid. This material could be deposited on the surface of the extracted cells to render them gram-positive again, but could not be deposited on normally gram-negative bacteria. The ribonucleate appeared to be highly specific in that related compounds or other salts of ribonucleic acid could not be substituted for it. This work has been confirmed and extended by Bartholomew and Umbreit[7] who converted gram-positive bacteria to gram-negative forms by digestion with ribonuclease and, further, were able to replace the bacterial ribonucleate with magnesium ribonucleate prepared from yeast. There was also evidence that the sulfhydryl groups of the protein were involved in some way in the gram reaction as a whole.

[3] Benians: Jour. Path. Bact., 1912, 17:199; *ibid.*, 1920, 23:401.

[4] Stearn and Stearn: Jour. Bact., 1924, 9:463, 479.

[5] Churchman: Jour. Exp. Med., 1927, 46:1009; Jour. Bact., 1929, 18:413; also in Jordan and Falk: *Newer Knowledge of Bacteriology and Immunology*. University of Chicago Press, Chicago. 1928.

[6] Henry and Stacy: Nature, 1943, 151:671.

[7] Bartholomew and Umbreit: Jour. Bact., 1944, 48:567. This paper includes a critical review of the pertinent literature.

The gram reaction, therefore, appears to depend on a surface structure of magnesium ribonucleate combined with protein which is present on gram-positive bacteria but lacking on gram-negative bacteria. This explanation is consistent also with the evidence brought forward by others but interpreted differently; for example, the reaction between the crystal violet and iodine reagents and the ribonucleate-protein complex does not occur at acid reactions, hence the reversal of the gram-positive reaction by lowering pH.

Procedure of the Gram Stain. In addition to crystal violet and safranin staining solutions, Lugol's iodine solution is required as a mordant. Its composition is:

iodine	1 gm.
potassium iodide	2 gm.
distilled water	300 ml.

The staining procedure is as follows:
(1) The heat-fixed smear is stained for 1 minute with ammonium oxalate crystal violet.
(2) Wash in tap water.
(3) Flood with Lugol's iodine solution and allow to stand 1 minute.
(4) Wash in tap water and blot dry.
(5) Decolorize 30 seconds with gentle agitation in 95 per cent alcohol, and blot dry.
(6) Counterstain 10 to 30 seconds in safranin.
(7) Wash in tap water, blot dry and examine.

Acid-Fast Stain (Ziehl-Neelsen Method). Certain bacteria, characterized by a high lipid content, cannot be stained by the usual procedure of the simple stain and either heat or prolonged contact is required to drive the stain into the cells. Conversely, the stained forms are equally difficult to decolorize and resist decolorization with acid alcohol. These organisms are designated acid-fast and include the tubercle bacilli and related forms, the leprosy bacillus and certain of the actinomycetes.

Staining Procedure:
(1) Stain the smear for 3 to 5 minutes in steaming Ziehl's carbol fuchsin. An alternative procedure useful in diagnostic laboratories is to stain in the cold for 18 to 24 hours.
(2) Rinse in tap water.
(3) Decolorize in 95 per cent ethyl alcohol, containing 3 per cent by volume of concentrated hydrochloric acid, until only a suggestion of pink color remains.
(4) Wash in tap water.
(5) Counterstain for 30 to 60 seconds with alkaline methylene blue.
(6) Wash in tap water, dry and examine.

Fontana Stain for Spirochetes. The spirochetes stain very poorly or not at all by the usual simple staining procedure, and are usually stained by a silver impregnation method.

Preparation of Ammoniacal Silver Nitrate Solution. Dissolve 5 gm. silver nitrate in 100 ml. of distilled water. Remove a few ml., and to the rest of the solution add, drop by drop, concentrated ammonia solution until the sepia precipitate which forms is dissolved. Then add, drop by drop, enough more of the silver nitrate solution to produce a slight cloud which persists after shaking. This solution is stable for some months.

Staining Procedure:
(1) Steam the heat-fixed smear in a solution of 5 per cent tannic acid in 1 per cent phenol for 30 seconds.
(2) Wash for 30 seconds in running water.
(3) Flood with ammoniacal silver nitrate solution, heat gently, and allow to stand 20 to 30 seconds after steaming has begun.
(4) Wash in tap water, blot dry and examine.
The spirochetes appear as dark brown or black on a dark maroon field.

Capsule Stain (Hiss's Method). The bacterial capsule does not stain in the simple stain or Gram stain procedures. A number of methods of staining the capsules have been used of which that of Hiss is one of the most simple and effective. The stain is an aqueous solution of basic fuchsin or crystal violet, 0.15 to 0.3 per cent and 0.05 to 0.1 per cent respectively.

Staining Procedure. The bacteria should be grown in ascitic fluid or serum medium for maximum capsule development, and bacteria grown on solid media should be suspended in serum for preparation of the smear. The smear is air dried and fixed by heat.

(1) Stain with either aqueous basic fuchsin or aqueous crystal violet by heating gently until the stain steams.

(2) Wash off the stain with 20 per cent aqueous copper sulfate solution.

(3) Blot dry and examine.

The bacterial cells are deeply stained and the capsules a faint blue or pink, depending upon which stain has been used.

Spore Stain. Like acid-fast bacteria, spores are very difficult to stain and appear as unstained in the usual stained smear. The stain may be driven in by heat, and the Ziehl-Neelsen acid-fast staining procedure may be used with the modification that decolorization is less rigorous, *i.e.*, 95 per cent alcohol or absolute acetone should be used instead of acid alcohol.

CULTURE OF BACTERIA

Methods of Obtaining Pure Cultures. When fluid culture media are inoculated with substances such as soil, water or excreta, many kinds of organisms develop simultaneously side by side, and a heterogeneous mixture, or mixed culture, of bacteria results. Any technical procedure for obtaining such pure cultures is dependent upon the isolation of a single viable bacterium which is allowed to multiply in a suitable culture medium. The first reliable method of isolation of pure cultures was devised by Koch in 1881. This method has proved so satisfactory that it has been employed to the present day with only minor modifications. If nutrient gelatin or agar is inoculated while fluid (for example, at 42° C.) and is then solidified and kept under favorable temperature conditions, many of the living bacteria that have been introduced are able to multiply. Since the bacteria cannot move about freely, but are fixed in the stiffened medium, the progeny of each organism form distinct masses or colonies. These colonies consist of many millions of bacteria and are readily visible to the naked eye or by means of a low power hand lens. If the colonies are not closely crowded, a pure culture may be obtained by touching a colony with the tip of a sterile needle and inoculating tubes of fresh culture media. In order to secure a large surface upon which the colonies shall be spread out and made easily accessible, the gelatin or agar after inoculation is poured while still fluid into sterilized flat shallow dishes (petri dishes) fitted with glass covers.

It will be clear that a given colony may arise from two or more parent cells if these remain attached or close together in the agar medium, and the colony will not be a pure culture in the event that the juxtaposed cells are of different species. This possibility has been investigated by McNew[8] using the plant pathogen *Phytomonas stewarti.* He found that 98 per cent or more of the colonies arose from single cells, hence the probability of a mixed culture in a single colony is 0.02 or less. Successive plating or picking a number of colonies

[8] McNew: Phytopathology, 1938, 28:387.

makes a pure culture a practical certainty. It is likely, however, that this figure differs for different bacteria, *i.e.*, staphylococci are more likely to remain attached than micrococci or bacilli.

It is self-evident that the pure culture so obtained is not only homogeneous with respect to kind or species of bacteria, but since the microorganisms are descendants of a single parent cell, they constitute what the zoologist or pro-tozoologist terms a *clone*.

Technique of Making Plate Cultures. Three tubes of agar (1, 2, 3), melted at 100° C., are placed in a water bath at 42° C., a temperature that is just above the solidifying point of agar and is not injurious to bacteria. Tube 1 is inoculated with a loopful of the material to be plated. The cotton plug is then replaced and the contents of the tube are mixed by carefully tilting back and forth and rotating the tube on its long axis. From this tube two loopfuls of agar are transferred to tube 2, and after mixing, two more loopfuls carried from tube 2 to tube 3. The contents of the several tubes are then poured into petri dishes. As soon as the cotton plug is removed, the mouth of each tube should be passed through the flame and inserted under the edge of the lifted petri dish cover, and the agar quickly poured out. The covered petri dish may then be tipped cautiously back and forth to dis-tribute the agar evenly before it solidifies. Agar plates placed in the incubator after solidi-fication should be inverted in order to avoid spreading of the growth through condensation of moisture on the surface of the medium. Even if there are a great many bacteria in the original material, the plate from tube 3 will probably contain the organisms in small enough numbers to develop well isolated colonies. On the other hand, if there are very few bacteria in the material inoculated, plate 1 will probably present more satisfactory conditions. Gela-tin plates are made in the same manner as agar except that gelatin may be cooled as low as 25° C. without solidifying.

Under exceptional conditions, such as work in the field, when petri dishes are not available, so-called "roll tubes" may be made. The tubes containing the liquid agar medium are tilted until the agar almost reaches the cotton plug. The tubes are then rotated in this position against a block of ice, and when the process is complete the test tube is coated on the inside with a thin layer of solid medium. In this way a considerable surface is ob-tained and, after incubation, colonies are readily picked.

Quantitative Dilution. It is sometimes of advantage before plating to make accurate dilutions of highly polluted fluids, such as sewage, in order to get colonies few enough in number to be well isolated. If there is reason to suppose that the number of bacteria is 200 per ml. or more, 1 ml. of the sample is mixed with 9 ml. of sterile water. If a higher dilution is required, proceed in a similar manner.

(A) To dilute 1:		10	use	1 ml.	of sample	to	9 ml.	of sterile water.
(B)	" 1:	100	"	"	"	" 99	"	"	"
(C)	" 1:	1,000	"	"	(A)	" 99	"	"	"
(D)	" 1:	10,000	"	"	(B)	" 99	"	"	"
(E)	" 1:	100,000	"	"	(C)	" 99	"	"	"
(F)	" 1:	1,000,000	"	"	(D)	" 99	"	"	"

One milliliter of each dilution is placed in a sterile petri dish and 6 to 8 ml. of liquid cooled agar medium are added. The two are thoroughly mixed by tilting the petri dish back and forth and then allowed to solidify. After incubation the total number of colonies on the plates containing 50 to 200 colonies is counted and the total number is multiplied by the dilution to give the number of organisms present in the original sample. Such bacterial counts are relatively inaccurate, errors of 10 to 15 per cent being common in even the most careful work, and must be regarded only as useful approximations.

Streak Plates. Not infrequently media used for the isolation of the more fastidious bacteria are such that it is technically difficult to make the usual pour plates. The most common of these media are those which contain fresh blood. Such media are ordinarily prepared in quantity and sterile plates poured. These may be stored in the ice-box until

used. Dilution and consequent isolation of bacteria may be accomplished with these media by a process known as streaking. A sterile wire loop is dipped into the bacterial suspension and rubbed back and forth on the surface of the solid medium, making successive streaks as close together as possible. After incubation it will be found that at some point sufficient numbers of bacteria were rubbed off the loop to allow the development of individual colonies in the path of the streak. These may be picked and transferred to fresh media. Quantitative dilutions may be prepared in sterile broth or saline and 0.5 ml. of each dilution pipetted on to the surface of the medium and spread evenly by appropriate tilting.

Carbon Dioxide Tension. A number of bacteria, particularly the gonococcus, meningococcus and Brucella, require an increased tension of carbon dioxide over that of ordinary atmosphere, commonly 10 to 15 per cent. Cultures of such bacteria are incubated in air-tight jars in which a part of the air has been replaced by carbon dioxide. One or another of three methods is commonly used.

(1) The most obvious and effective method is direct replacement in which the jar is a vacuum desiccator that is partially evacuated, 9 to 10 cm. Hg negative pressure, and the evacuated air replaced with carbon dioxide from a tank of the compressed gas. The method has the disadvantage that it requires a certain amount of equipment.

(2) A simple and equally effective method is that of putting a short candle stub in the jar with the cultures, lighting it and closing the jar. The candle burns for a short time, forming carbon dioxide, and then goes out. The partial pressure of carbon dioxide obtained is not subject to control but the precise tension is not too important and adequate amounts are formed.

(3) Carbon dioxide may also be generated from sodium bicarbonate by the addition of sulfuric acid. The amounts required must be calculated on the basis of the size of the jar used so that 10 to 15 per cent of the total gas volume is the evolved carbon dioxide.

Cultivation of Obligate Anaerobes. Atmospheric oxygen is toxic to a group of bacteria designated the obligate anaerobes, which includes the bacilli of tetanus and gaseous gangrene, the botulinus bacillus, the Bacteroides and anaerobic cocci. Various methods have been devised for the cultivation of these bacteria which center about the elimination of gaseous oxygen and which may be grouped under four general heads.

(1) *Enriched Media in Deep Tubes.* Perhaps the simplest method is that of the so-called shake culture which is in an agar medium liquefied and boiled to drive out dissolved oxygen, inoculated when cool but still liquid, and solidified quickly in cold water. This is the basis of culture in the Veillon tube, a glass tube stoppered at both ends, which is sometimes used in the isolation of obligate anaerobes in pure culture; the tube may be broken or the column of agar forced out into a sterile petri dish and cut at the various points where isolated colonies occur deep in the medium.

A somewhat simpler method of culture in liquid medium is based on the use of a rich medium to which is added a piece of fresh sterile tissue such as rabbit kidney. Such media show strong oxygen uptakes as a result of the respiration of the fresh tissue, and the depths of the medium are anaerobic. As soon as growth is initiated the evolution of gas, usually from contained glucose, is vigorous enough to prevent solution of atmospheric oxygen and the culture remains anaerobic.

(2) *Removal of Oxygen by Combustion.* Oxygen may be removed from the gaseous environment of cultures incubated in sealed jars in a number of ways. Sufficiently anaerobic conditions can frequently be obtained by saturating a piece of filter paper with alcohol, placing in the jar and igniting, and sealing the jar immediately. Similarly, a small metal container filled with chalk may be put into a jar, a piece of phosphorus placed in it and the jar sealed immediately. The phosphorus pentoxide formed will dissolve in a small amount of water placed in the bottom of the jar and is not harmful to bacterial cultures, but the

phosphorus begins to burn again when the jar is opened and the method is somewhat dangerous.

By far the most convenient combustion method is that in which the oxygen is removed by combination with hydrogen in the presence of heated platinized asbestos. Jars designed for this purpose with platinized asbestos heated by a small electric coil have been described by Brewer and are termed Brewer anaerobic jars. The jar rim is sealed against both positive and negative pressure and hydrogen or illuminating gas run in to give some positive pressure. The electric current is turned on and the oxygen is burned out in the presence of the combustible gas and catalyst. This method is considerably more reliable than the combustion methods described above.

(3) *Chemical Absorption of Oxygen.* Contained oxygen in sealed jars may also be removed by chemical methods, of which the most widely used is a mixture of pyrogallic acid and alkali in the bottom of a vacuum desiccator. The jar should be closed immediately on addition of the reagents and it is preferable to mix after closing by tilting or spilling one reagent into the other. Excess alkali is to be avoided. The reagents are 20 per cent aqueous sodium hydroxide and 40 per cent aqueous pyrogallic acid mixed in proportions of 5 ml. of alkali to 2 ml. of pyrogallic acid. The alkali-pyrogallic acid method is applicable to single culture tubes; a stopper of absorbent cotton is pushed down to leave about an inch of space above it, pyrogallic acid and sodium hydroxide placed in this space to saturate the cotton, the tube closed at once with a tight-fitting rubber stopper and turned upside down and incubated in that position. It may be used also for the anaerobic incubation of a single petri dish in combination with the Spray dish, which is a false bottom, so to speak, over which the medium-containing half of the petri dish is inverted and sealed. The Spray dish is divided by a ridge in the center; 4 ml. pyrogallic acid is put on one side and 10 ml. of alkali on the other; after sealing on the petri dish bottom, the two are mixed by tilting.

(4) *Displacement of Air by an Inert Gas.* The air contained in an anaerobic jar may be mechanically displaced by an inert gas, commonly hydrogen since tanked nitrogen contains sufficient oxygen to inhibit the growth of the fastidious obligate anaerobes. A jar with two connections, one reaching to the bottom, designated a Novy jar, may be used with tanked hydrogen. The hydrogen is run in from the bottom of the jar until the contained air is displaced. The air in an anaerobic jar may also be displaced by hydrogen evolved in the reaction between sulfuric acid and powdered chromium metal.

Control of Anaerobiosis. It is usually desirable to include an indicator of anaerobiosis in an anaerobic jar to indicate that anaerobic conditions have been obtained and are maintained, *i.e.*, there is no leakage of atmospheric oxygen into the jar during incubation. The usual indicator is a tube of methylene blue prepared as follows: equal parts of N/160 sodium hydroxide, 0.015 per cent methylene blue and 6 per cent sterile aqueous solution of glucose are mixed, boiled just before the jar is to be sealed until the methylene blue is reduced, and the open tube put into the jar. So long as it remains colorless anaerobic conditions are maintained.

SYSTEMATIC STUDY OF BACTERIA IN PURE CULTURE

The characteristics of bacteria that are of significance in their identification must be studied in some uniform fashion, not only with regard to the methods used but also as to the characteristics that shall be considered of first importance. Although the classification of bacteria is in a somewhat dubious state, this uncertainty has resulted largely from a relatively hazy comprehension of the fundamental biological significance of some differential characteristics and consequent doubt as to the proper relative weight to be assigned to them in the arrangement of the finer subdivisions of taxonomic schemes. The main lines of approach are, however, clear, and whatever their relative biological significance may prove to be, the readily determinable characteristics of bacteria serve the practical purpose of distinguishing these microorganisms.

There are four general categories which, in the order of the fineness of distinction they make possible, are: (*a*) morphology, both gross and microscopic; (*b*) physiologic capabilities in terms of biochemical reactions; (*c*) pathogenicity for experimental animals; (*d*) immunologic character.

The preliminary and basic study of a bacterium lies in the systematic determination of its cultural characters. The most important of these are:

(*A*) Morphology
    1. gross morphology—that of colonies of the organism with respect to size, texture, color, shape, etc.
        (*a*) on nutrient or infusion semisolid media
        (*b*) on special media
    2. microscopic morphology, including
        (*a*) size, shape and grouping of the organisms
        (*b*) presence or absence of spores
        (*c*) motility
        (*d*) presence or absence of capsule
        (*e*) staining reactions
(*B*) Biochemical reactions
    1. the fermentation of sugars, usually dextrose, lactose and sucrose, although others may be included together with the hydrolysis of starch
    2. liquefaction of gelatin
    3. formation of indol
    4. reduction of nitrate to nitrite
    5. production of hydrogen sulfide
    6. special biochemical tests such as the Voges-Proskauer reaction, the methyl red test, hemolysis on blood agar, etc.

Such preliminary examination ordinarily affords a great deal of information about a given bacterial culture. The feasibility of additional study rests firmly on the foundation laid down by the studies outlined above.

Various attempts have been made to systematize the study of bacteria. One of the most successful of these has resulted from the efforts of a Committee on Bacteriological Technique of the Society of American Bacteriologists, under whose auspices a Manual of Pure Culture Study has been published and is supplemented by leaflets from time to time. Through this agency standard methods and descriptive terminology are made available. Furthermore, the same committee has prepared a standard descriptive chart for the recording of data, and from time to time the chart is revised.

ANIMAL INOCULATION

An indispensable adjunct to the study of the pathogenic bacteria is the experimental animal. Animals are used not only for the study of the pathology of infectious disease and as an aid in the isolation of some bacteria in pure culture, but also for the experimental production of immune sera and studies on the various manifestations of immune phenomena. The maintenance of such infectious agents as the filterable viruses, which cannot be cultivated on lifeless media, by animal passage is common also. The animals generally used are rabbits, guinea pigs, white mice and white rats, although in special cases others, such as rhesus monkeys, are necessary.

Routes of Inoculation. Experimental animals may be inoculated by a variety of routes, usually one or another being preferable under the particular cir-

Name of organism .... Source .... Studied by ....

Date of isolation .... Habitat .... Optimum conditions: Media .... Culture No. ....Temp. ....°C.

Is phase variation observed? .... Phase on this Chart: S, R, M, G (smooth, rough, mucoid, gonidial) .... Phases recorded on other charts: ....

SKETCHES

Underscore required terms.

VEGETATIVE CELLS: Medium used.....................................
Reaction (pH).....................................Temp.....................................Age.....................................d
Size (μ): Majority.....................................
Ends, rounded, truncate, concave, tapering.

MOTILITY: In broth.....................................On agar.....................................
SPORANGIA AND ENDOSPORES: present, absent.
Medium used.....................................pH.....................................Temp.....................................Age.....................................d
Endospore Form: spherical, ellipsoid, cylindrical
IRREGULAR FORMS:
Present in.....................................days at.....................................°C.

AGAR COLONIES: Temperature.....................................°C. Age.....................................d.
Form, punctiform (i. e. under 1 m.m. diam.), circular (i. e. over 1 m.m. diam.), filamentous, irregular, rhizoid.
Surface, smooth, rough, concentrically ringed, radiately ridged.
Edge, entire, undulate, lobate, erose, filamentous, curled.
Elevation of growth, effuse, flat, raised, convex.
Optical Characters, opaque, translucent, opalescent, iridescent.

GELATIN COLONIES: Temperature.....................................°C. Age.....................................d.
Form, punctiform, circular, filamentous.
Elevation, flat, raised, convex, pulvinate, crateriform (liquefying).
Edge, entire, undulate, lobate, erose, filamentous, curled.
Liquefaction, crateriform, saucer, spreading.
Surface, smooth, contoured, rugose.
Optical Characters, opaque, translucent, opalescent, iridescent.

AGAR STROKE: Temperature.....................................°C. Age.....................................d.
Growth, scanty, moderate, abundant, none, beaded, spreading.
Form of growth, filiform, echinulate, beaded, spreading, arborescent, rhizoid.
Lustre, glistening, dull.
Chromogenesis.....................................photogenic, fluorescent.
Odor, absent, decided, resembling.
Consistency, butyrous, viscid, membranous, brittle.
Medium, grayed, browned, reddened, blued, greened, unchanged.

NUTRIENT BROTH: Temperature.....................................°C. Age.....................................d.
Surface growth, ring, pellicle, flocculent, membranous, none.
Clouding, slight, moderate, strong, transient, persistent, none.
fluid turbid, granular growth.
Odor, absent, decided, resembling.
Sediment, compact, flocculent, granular, flaky, viscid.
Amount of sediment, abundant, scanty, none.

GELATIN STAB: Temperature.....................................°C. Age.....................................d.
Growth, uniform, best at top, best at bottom.
Line of puncture, filiform, beaded, papillate, villous, arborescent.
Liquefaction, none, crateriform, infundibuliform, napiform, saccate, stratiform; begins in.....................................d.
Degree of liquefaction in.....................................days; complete in.....................................d.
Method used.....................................
Medium, fluorescent, browned, unchanged.

	Surface Colonies	Deep Colonies
	Surface Colonies	Deep Colonies
	Medium: Temperature.....°C. Age.....d.	
	Medium: Temperature.....°C. Age.....d.	
	Medium: Temperature.....°C. Age.....d.	

FERMENTATION Temperature.....................................°C.

	Monosaccharides	Disaccharides	Polysaccharides	Alcohols	Glucosides
Medium....................	Arabinose / Rhamnose / Xylose / Glucose / Fructose / Galactose / Mannose	Lactose / Sucrose / Maltose / Trehalose / Melibiose / Cellobiose	Raffinose / Melezitose / Starch / Inulin / Dextrin / Glycogen	Glycerol / Erythritol / Arabitol / Adonitol / Mannitol / Sorbitol / Dulcitol	Salicin / Aesculin / Coniferin / a-Methyl Gluc.
containing....................					
.....................and:					

Gas in fermentation tube
Amt. CO₂ in Eldredge tube
Reaction (pH) after.....................................d.
Titrable acidity in ml. of N/.....................................NaOH

BRIEF CHARACTERIZATION

As each of the following chief characteristics is determined, indicate in proper marginal square by means of figure, as designated below. In case any of these characteristics are doubtful or have not been determined, indicate with the letters U, V, and X according to the following code: U, undetermined; V, variable; X, doubtful.

MORPHOLOGICAL — VEGETATIVE CELLS	Form & arrangement: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinae; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous
	Diameter: 1, under 0.5μ; 2, between 0.5μ and 1μ; 3, over 1μ
	Gram stain: 0, negative; 1, positive
	Flagella: 0, absent; 1, peritrichic; 2, polar; 3, present but undetermined
	Capsules: 0, absent; 1, present
	Chains (4 or more cells): 0, absent; 1, present
	SPORANGIA: 0, absent; 1, elliptical; 2, short rods: 3, spindled; 4, clavate; 5, drumstick
	ENDOSPORES: 0, absent; 1, central to excentric; 2, subterminal; 3, terminal
CULTURAL — AGAR STROKE	Growth: 0, absent; 1, abundant; 2, moderate; 3, scanty
	Lustre: 1, glistening; 2, dull
AGAR COLONIES	Form: 1, punctiform; 2, circular (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled; 6, irregular
	Surface: 1, smooth; 2, contoured; 3, rugose
GELATIN COLONIES	Form: 1, punctiform; 2, circular (over 1 mm.); 3, irregular; 4, filamentous
	Surface: 1, smooth; 2, contoured; 3, rugose
PHYSIOLOGICAL	Biologic relationships: 1, pathogenic for man; 2, for animals but not for man; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic
	Relation to free oxygen: 1, strict aerobe; 2, facultative anaerobe; 3, strict anaerobe; 4, microaerophile
	In nitrate media: 0, neither nitrite nor gas; 1, both nitrite and gas; 2, nitrite but no gas; 3, gas but no nitrite
	Chromogenesis: 0, none; 1, pink; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, black
	Other photic characters: 0, none; 1, photogenic; 2, fluorescent; 3, iridescent
	Indole: 0, negative; 1, positive
	Hydrogen sulfide: 0, negative; 1, positive
	Hemolysis: 0, negative; 1, positive
	Methemoglobin: 0, negative; 1, positive
PROTEID LIQUEFACTION OR DIGESTION	Gelatin: 0, negative; 1, positive
	Casein: 0, negative; 1, positive
	Egg albumin: 0, negative; 1, positive
	Blood serum: 0, negative; 1, positive
INDICATOR REDUCTION	Litmus: 0, negative; 1, positive
	Methylene blue: 0, negative; 1, positive
	Janus green: 0, negative; 1, positive
	Rennet production: 0, negative; 1, positive

SUPPLEMENTARY DATA

TEMPERATURE RELATIONS

Medium..................................pH..................

Optimum temperature for growth..................°C.

Maximum temperature for growth..................°C.

Minimum temperature for growth..................°C.

THERMAL DEATH POINT: Time 10 minutes:..................°C.

Medium..................................pH..................

THERMAL DEATH TIME:

Medium..................................pH..................

Temp.	Time	Temp.	Time
..........°C.	..........min.	..........°C.	..........min.
..........°C.	..........min.	..........°C.	..........min.
..........°C.	..........min.	..........°C.	..........min.

CHROMOGENESIS

Gelatin..................................

Agar..................................

Potato..................................

OTHER PHOTIC CHARACTERS

Photogenesis on..................................

Iridescence on..................................

Fluorescence in..................................

RELATION TO REACTION (pH) OF MEDIUM

Medium..................................

Optimum for growth: about pH..................

Limits for growth from pH..................to..................

RELATION TO FREE OXYGEN

Method..................................

Medium..................................Temp..........°C.

Aerobic growth: *absent, present, better than anaerobic growth; microaerophilic*

Anaerobic growth: *absent, occurs in presence of glucose, of sucrose, of lactose, of nitrate; better than aerobic growth*

Additional data..................................

MILK

Temperature..........°C.

Reaction:	..........d. ....;
Acid curd:	..........d. ....;
Rennet curd:	..........d. ....;
Peptonization:	..........d. ....;

LITMUS MILK

Temperature..........°C.

Reaction:	..........d. ....;
Acid curd:	..........d. ....;
Rennet curd:	..........d. ....;
Peptonization:	..........d. ....;
Reduction of litmus begins in.......days, ends in.......days

ACTION ON ERYTHROCYTES

Cells..................................

Method: *plate, broth, filtrate*

Hemolysis: *negative, positive*

Methemoglobin: *negative, positive*

PRODUCTION OF INDOLE

Medium..................................

Test used..................................days

Indole *absent, present in*..................

PRODUCTION OF HYDROGEN SULFIDE

Medium..................................

Test used..................................

H₂S *absent, present in*..................days

ACTION ON NITRATES

Medium..................................Temp..........°C.

Nitrite: ......d. ....; ......d. ....; ......d. ....; ......d.

Gas (N₂): ......d. ....; ......d. ....; ......d. ....; ......d.

Medium..................................Temp..........°C.

Nitrite: ......d. ....; ......d. ....; ......d. ....; ......d.

Gas (N₂): ......d. ....; ......d. ....; ......d. ....; ......d.

Ammonia production (in amino-N-free medium):

negative, positive..................medium:

Complete disappearance of nitrate in..................medium:

negative, positive

Disappearance of 2 p.p.m. nitrite in..................medium:

negative, positive

REDUCTION OF INDICATORS

Medium..................pH..........Temp..........°C.

Indicator	Conc.	Reduction:
....	......%	.......hr. ....; ....hr.
....	......%	.......hr. ....; ....hr.
....	......%	.......hr. ....; ....hr.

STAINING REACTIONS

Gram: ......d. ....; ......d. ....; ......d.

Method..................................

Spores: Method..................................

Capsules: Method..................................

Medium..................................

Flagella: Method..................................

Special Stains..................................

ADDITIONAL TESTS

Methyl red: *negative, positive*

Voges-Proskauer: *negative, positive*

Growth in sodium citrate: *absent, present*

Growth in uric acid: *absent, present*

Hydrolysis of starch: *complete (iodine colorless); partial (iodine reddish-brown); none (iodine blue)*

Nitrogen obtained from the following compounds:..................

....

PATHOLOGY

SPECIAL TESTS

ANIMAL INOCULATION

Medium used..................Age of culture..................Amount..................Incubation period..................

Animal		Whole culture	Cells	Filtrate
	Subcutaneous	*		
Type of Injection	Intraperitoneal			
	Intravenous			
	Per os			

*In each instance where pathogenicity is observed, indicate location of lesion, and type, e.g. edema, histolysis, gas, hemorrhage, ulcer, diphtheritic, etc.

ANTIGENIC ACTION

Animal..................Medium used..................Age of culture..................

Type injection..................Number of injections..................

Culture causes production of *cytolysins, agglutinins, precipitins, antitoxin.*

Specificity: Antibodies produced effective against other antigens as follows..................

Immune sera from..................effective against this organism as antigen

Name of organism...Studied by..........................Culture No..............

Source..Habitat.............................Date...................

Descriptions (*Underscore required terms.*)	Sketches

CELL MORPHOLOGY   Medium: Temp.   °C.

Vegetative cells: Age:
  Form and arrangement: *streptococci, diplococci, micrococci, sarcinae, rods' commas, spirals, branched rods, filaments.*
  Motility in broth: Flagella:
  Size: Irregular forms:

Sporangia: *none, rods, spindles, elliptical, clavate, drumstick.*   Age:
Endospores:
  Shape: *spherical, ellipsoid, cylindrical.*
  Position: *central to excentric, terminal, subterminal.*

STAINING CHARACTERISTICS

Gram: **Age:**   Method:
Special stains:

AGAR STROKE   Age: Temp.   °C.

Amount of growth: *scanty, moderate, abundant.*
Form: *filiform, echinulate, beaded, spreading, rhizoid.*
Consistency: *butyrous, viscid, membranous, brittle.*
Chromogenesis: ; *fluorescent, iridescent, photogenic.*

AGAR COLONIES   Age: Temp.   °C.

Form: *punctiform, circular, filamentous, rhizoid, irregular.*
Elevation: *effuse, flat, raised, convex.*
Surface: *smooth, contoured, radiate, concentric, rugose.*
Margin: *entire, undulate, erose, filamentous, curled.*
Density: *opaque, translucent.*

NUTRIENT BROTH   Age: Temp.   °C.

Surface growth: *none, ring, pellicle, flocculent, membranous.*
Subsurface growth: *none, turbid, granular.*
Amount of growth: *scanty, moderate, abundant.*
Sediment: *none, granular, flocculent, viscid, flaky.*

GELATIN STAB   Age: Temp.

Liquefaction: *none, crateriform, infundibuliform, napiform, saccate, stratiform.*
Rate: *slow, moderate, rapid.*

OTHER MEDIA   Age: Temp.

PERMENTATION Temp. °C.					
Medium:		Glucose	Lactose	Sucrose	
Carbohydrate: % Indicator:					
Acid in days					
Acid in days					
Gas in days					
Gas in days					

ACTION ON MILK	Temp. °C.				
Indicator:	Days				
Reaction					
Acid curd					
Rennet curd					
Peptonization					
Reduction (before coagulation)					

ACTION ON NITRATES

    Medium: Temp.   °C.

    Nitrite: . . . . . . .d.   ; . . . . . . .d.   ; . . . . . .d.

    Gas (N): . . . . . . .d.   ; . . . . . . .d.   ; . . . . . . .d.

HYDROGEN SULFIDE PRODUCTION

    Medium: Age:

    H₂S: *present, absent.*   Temp.   °C.

TEMPERATURE RELATIONS

    Growth in refrigerator (°C.): *present, absent.*

    Growth at room temperature (°C.): *present, absent.*

    Growth at 37°C.: *present, absent.*

    Growth at 50°C.: *present, absent.*

INDOLE PRODUCTION

    Medium: Age:

    Method: Temp.   °C.

    Indole: *present, absent.*

RELATION TO FREE OXYGEN

    Medium: Age:

    Method: Temp.   °C.

    Aerobic growth: *absent, present, better than anaerobic growth, poorer than anaerobic growth.*

    Anaerobic growth: *present, absent.*

ADDITIONAL TESTS

cumstances. The common routes of inoculation are intradermal, subcutaneous, intramuscular, intraperitoneal and intravenous. Other routes such as intra-ocular, intracerebral, intrathecal, etc., may be desirable at times. In any case, inoculation is carried out with a syringe and hypodermic needle, the capacity of the syringe and size of the needle used depending upon the quantity of material to be injected and the route of inoculation. The site of inoculation must be prepared by removal of the hair by shaving or a depilatory, followed by disinfection by swabbing with alcohol, tincture of iodine, etc. The syringe and needles have, of course, been previously sterilized by dry heat or by boiling.

Intracutaneous Inoculation. Material inoculated intracutaneously is inoculated into the skin. A fold of skin is pinched up and the needle inserted, lumen up, as superficially as possible. A raised white spot showing the pits of the hair follicles indicates a successful injection. Not more than 0.2 ml. may be inoculated by this route.

Subcutaneous Inoculation. Subcutaneous inoculation requires considerably less skill on the part of the operator. A fold of skin is pinched up as before and the needle inserted through the skin to its full length. The injected material forms a bleb or blister. The amount that may be injected by this route depends upon the size of the animal.

Intramuscular Inoculation. In the intramuscular inoculation, the needle is inserted deep into the muscular tissue, usually the posterior muscles of the thigh or the lateral thoracic or abdominal muscles.

Intraperitoneal Inoculation. Intraperitoneal inoculation is carried out as one would a subcutaneous injection on the abdomen except that after the needle penetrates the skin, it is held at right angles to the peritoneal wall and thrust through into the peritoneal cavity.

Intravenous Inoculation. Intravenous inoculation may be carried out in a variety of ways, the choice depending upon the size of the animal. Rabbits are conveniently inoculated in the marginal vein of the ear. The needle is inserted through the skin and into the vein from the side. If the needle is within the vein, blood will disappear in the vein from the point of the needle onward as the material is injected. Needless to say, the injection should take place in the direction of blood flow. Guinea pigs may be inoculated into the large super-ficial vein on the dorsal and inner side of the hind leg or into one of the external jugular veins. Both require anesthesia of the animal and a small incision, which may be later closed with a stitch or collodion. Rats may be inoculated in an external jugular vein and mice in one of the lateral veins of the tail.

Drawing Blood. Blood may be taken from the rabbit from the marginal vein of the ear or directly from the heart. The appropriate area is shaved and swabbed with alcohol. If blood is to be taken from the ear, the marginal vein is cut across with a razor blade and the blood allowed to drop into a centrifuge tube or test tube. Flow may be hastened by pre-liminary application of a towel wrung out of hot water to induce hyperemia. When blood is to be taken directly from the heart, a 30 to 50 ml. syringe is used and the needle inserted between the ribs at the point of maximum pulsation and thrust into the heart, taking care to injure the pericardium as little as possible. Blood is most conveniently taken from smaller animals by this means.

Postmortem Examination. The autopsy of experimental animals dead of infection is often highly desirable. Not only may cultures be taken, but the gross and microscopic pathology is usually of significance. The animal is fastened down on a board, ventral surface up. It is good practice to have the board in a larger enameled tray to reduce to a minimum the danger of spreading infectious material. When cultures are to be taken, the animal must be opened with sterile instruments. Usually several pairs of scissors and forceps and two or three scalpels are necessary. The site of inoculation is examined for ulcerative or other changes, and the hair and skin are disinfected with cresol or lysol.

The skin is incised from the pubis to the neck and cut at right angles at the ends of the

incision. The two flaps may be laid back by separation of the cutaneous from the under-lying muscular tissue with a scalpel. The condition of the subcutaneous tissue and of axillary and inguinal lymph glands may be noted. The peritoneal fluid may be cultured by searing a small area on the surface of the abdomen with a hot spatula and puncturing with a syringe and needle or a Pasteur pipette into which the fluid may be aspirated. The ab-dominal cavity is opened with fresh instruments and the flaps are laid back as above. The condition of the abdominal organs, such as spleen and liver, may be noted and cultures taken by first searing the surface and then either puncturing and aspirating fluid with a syringe and needle or a Pasteur pipette, or by removing a small piece of tissue and placing it in nutrient medium. Likewise, pieces of tissue may be removed and placed in fixing fluid for subsequent sectioning. Again with fresh instruments, the costal cartilages are cut to make a V-shaped incision and this flap is laid back over the head, exposing the thoracic cavity. Any gross pathology may be noted, cultures may be taken from heart's blood and elsewhere if desirable, and pieces of tissue removed for fixation and sectioning. The animal is disposed of by incineration.

It is obviously impossible to define postmortem procedure rigorously. The lymphatic system of guinea pigs dead of tuberculous infection will be of considerable interest. Those dying of diphtheritic toxemia or infection will show characteristic lesions of the adrenals. In some diseases the important postmortem findings may be in the brain and spinal cord. The routine of examination will be determined, therefore, by the particular disease under consideration or, if this is not known, by the symptoms exhibited by the animal before death.

A number of precautions must be observed in the autopsy of animals dead of infectious disease. If cultures are to be taken, fresh sterile instruments must be used at each stage. Instruments once used should be returned to the boiling water sterilizer or placed in a tray containing lysol or, if to be used again, laid down on the edge of the tray with all sharp edges and points inward. Extreme care must be taken to avoid disseminating infectious material. With certain highly contagious diseases such as tularemia, rubber gloves may be desirable. Boiling water sterilization (five minutes) of used instruments is sufficient except with the spore-forming organisms such as anthrax and tetanus, in which case the instru-ments should be allowed to stand in a solution of lysol or cresol.

IMMUNOLOGICAL METHODS

The immunological or serological reactions serve to differentiate bacteria on the basis of their constituent antigens. Such immunological differences are usually, though not always, subordinate to the biochemical differentiation of species and in general serve to subdivide species into immunological types or varieties. The immunological identification of an unknown microorganism is of considerable diagnostic utility, and is frequently of very great value in epi-demiological studies in tracing the source and dissemination of infection. Fur-thermore, serum may be tested against a known antigen to establish the presence of the homologous antibody and thus by inference the presence, im-mediate or past, of the microorganism or its products in the host, and there-fore serves as an indirect diagnostic procedure. The specific neutralization of toxin by antitoxin *in vivo* serves to measure antibody in serum or the circulat-ing antibody in the living host, and in the latter is a measure of antitoxic im-munity. Of the *in vitro* immunological reactions, those of agglutination of a bacterial or other discrete antigen, precipitation of a soluble antigen, and complement fixation in the presence of soluble or discrete antigen are of general utility.

Titration of Diphtheria Antitoxin by the Römer Method. The intradermal inocu-lation of diphtheria toxin in the rabbit produces a local erythema, and central necrosis with larger doses, which may be used in the assay of toxin, and of toxin in the presence of anti-toxin. The end point ordinarily taken is the smallest amount of toxin that, in a dose of 0.2

ml., will produce a zone of erythema 10 mm. in diameter 48 hours after inoculation. This is designated the minimal reacting dose or MRD, and the activity of a toxin may be meas ured as MRD per ml.

Such a titration measures the potency or toxicity of the toxin preparation, but not its combining power with respect to antitoxin. An expression of the activity of the toxin in the presence of antitoxin is the definition of the Lr dose. This is that amount of toxin which, when mixed with 1 unit of standard antitoxin, contains 1 MRD of unneutralized toxin. It will be clear that a toxin so standardized with respect to standard antitoxin can then be used to titrate an unknown antitoxin by using a constant amount of toxin mixed with serial dilutions of the unknown antitoxin, and testing of the mixtures for activity.

One application of this technique is the titration of the antitoxin content of the serum of persons, immunized, convalescent or normal. This will be considered here since it illustrates all of the principles involved. In studies on human immunity the usual procedure is to titrate the serum against an amount of toxin which will just give the end point reaction when mixed with 0.002 units of standard antitoxin; this is commonly spoken of as titration at the 1/500th level.

Standardization of Toxin at the 1/500th Level. Toxins vary widely in potency and combining power and no precise quantitative procedure may be given. The general model of the titration is as follows:
(1) Assume that a toxin whose L+ dose is known is available. Then the following preliminary titrations can be carried out:
    (a) The standard antitoxin is diluted so that it contains 1 unit per ml.
    (b) The toxin is diluted so that it contains 1 L+ dose per ml.
    (c) The toxin is titrated in dilutions of 1:100, 1:200, 1:300, up to 1:1000 against constant amounts of antitoxin, *viz.,* 1 ml. of diluted antitoxin is mixed with 1 ml. of each toxin dilution, incubated as indicated below, 0.2 ml. of each inoculated intradermally in the rabbit, and the reaction read in 48 hours. This titration determines the Lr dose of toxin.
    (d) Additional preliminary titration indicates the approximate amount of toxin required to just give the skin reaction in the presence of 1/500 unit of antitoxin. A more precise determination is carried out as follows:
      (i) Prepare a dilution of the original toxin in sterile broth to contain exactly 100 Lr per ml. This is the so-called stock solution and is relatively stable if prepared from stabilized (aged) toxin.
      (ii) Dilute 1 ml. of the stock solution of toxin with 99 ml. of buffer solution to give a solution containing 1 Lr per ml.
      (iii) Prepare serial dilutions at intervals of 5 within the limits indicated by the above preliminary titration. Thus, if the end point is indicated as lying between 1:40 and 1:80, prepare 1:40, 1:45, 1:50, up to 1:80.
      (iv) Mix 1 ml. of each dilution of toxin with 1 ml. of standard antitoxin diluted to contain 0.002 unit per ml. Incubate the mixtures for 1 hour at 37° C. and leave in the refrigerator overnight to allow ample time for the toxin-antitoxin reaction.
      (v) Inject 0.2 ml. of each dilution mixture intradermally into the shaven skin of a rabbit (as many as 40 such inoculations may be made in a single animal without ill effects) and identify the sites of inoculation with dye.
      (vi) Read at 48 hours, the end point being that dilution of toxin which produces a zone of erythema nearest to 10 mm. in diameter.

Titration of Antitoxin at the 1/500th Level. For the titration of antitoxin in serum proceed as follows:
(1) Dilute the stock solution of toxin as indicated in the above standardization, *i.e.,* so that 1 ml. contains sufficient toxin to give the skin reaction when mixed with 1 ml. of antitoxin containing 0.002 unit per ml. and inoculated in amounts of 0.2 ml.
(2) Prepare dilutions of patient's serum, say, for illustrative purposes, of 1:10 and 1:500.
(3) Mix 1 ml. of undiluted serum and 1 ml. of each of the dilutions with 1 ml. each of the diluted toxin, and in addition set up a control which contains 1 ml. of the diluted toxin and 1 ml. of standard antitoxin diluted to contain 0.002 unit.

(4) Incubate, inoculate, and read as above.

Suppose the toxin mixed with the 1:500 and 1:10 dilutions of serum gives a skin re-action, but that mixed with the undiluted serum does not. It follows that the undiluted serum contains more than 1/500 unit of antitoxin per ml. but it contains less than 1/50 unit per ml. since it is not neutralized by the 1:10 dilution of serum. By varying the serum dilution, any fraction of a unit may be determined as indicated in the accompanying table.

DILUTIONS OF PATIENT'S SERUM FOR TITRATION OF ANTITOXIN
AT 1/500TH LEVEL

Patient's Serum (ml.)	Saline (ml.)	Dilution	Amount Mixed with 1 ml. of Toxin Dilution (ml.)	Fraction of Antitoxin Unit Tested for
1.0	0	0	1	1/500
0.5	0.5	1 in 2	1	1/250
0.2	0.8	1 in 5	1	1/100
0.1	0.9	1 in 10	1	1/50
0.1	1.6	1 in 16.6	1	1/30
0.1	2.4	1 in 25	1	1/20
0.1	4.9	1 in 50	1	1/10
0.1	9.9	1 in 100	1	1/5
0.1	49.9	1 in 500	1	1/1

The Precipitin Reaction. The precipitin reaction is the specific forma-tion of a precipitate when a soluble antigen and its homologous antiserum are mixed. It is used for the identification of soluble antigens of bacterial extracts, such as pneumococcus polysaccharide, the polysaccharide and protein antigens of the streptococci, etc., and is used for the immunological typing of a number of kinds of bacteria. It is also used for the identification of other protein anti-gens as, for instance, the identification of bloodstains in forensic medicine.

In the precipitin test antiserum is used undiluted, or in very low dilution which avoids undesirable cross reactions, and is admixed with successive dilutions of the antigen. The titer of the antiserum is expressed as the highest dilution of antigen with which precipita-tion occurs. It is one of the most delicate and sensitive of the serological reactions; antisera which will precipitate an antigen such as pneumococcus polysaccharide in dilutions as high as 1:1 to 1:5 million are not uncommon, and titers to serum antigens are frequently 1:10,000 to 1:100,000. The test is carried out as follows:

(1) *Preparation of the Antigen Dilutions:*

    (a) Set up a series of small test tubes, such as Wassermann tubes, each of which contains 0.5 ml. of saline solution.

    (b) To the first tube add 0.5 ml. of the antigen solution, mix well by blowing in and out of the pipette several times, and transfer 0.5 ml. of this dilution to the second tube. The procedure is continued through as many tubes as the titer, or expected titer, of the serum indicates to give a series of dilutions of the antigen to 2^n. If the original antigen solution is not diluted, these dilutions will be 1:2, 1:4, 1:8, etc., but it is more convenient to prepare a 1:100 dilution of the orignial antigen to begin with, thus giving dilutions of 1:100, 1:200, 1:400, up to 1:25,600, etc.

(2) *Procedure of the Test:*

    (a) Distribute the antiserum in amounts of 0.1 ml. in a series of 5 x 50 mm. test tubes, as many as the dilution range to be observed requires.

PROTOCOL FOR THE PRECIPITIN TITRATION

Tube Number	Antiserum (ml.)	Antigen	
		Dilution	Amount (ml.)
1	0.1	1:100	0.1
2	0.1	1:200	0.1
3	0.1	1:400	0.1
4	0.1	1:800	0.1
5	0.1	1:1600	0.1
6	0.1	1:3200	0.1
7	0.1	1:6400	0.1
8	0.1	1:12,800	0.1
9	0.1	1:25,600	0.1
antiserum control	0.1	saline solution	0.1
antigen control	saline solution 0.1	undiluted antigen	0.1

(b) Carefully layer 0.1 ml. of the antigen dilutions onto the antiserum, each of the serial dilutions in successive tubes, so that the juncture between the two is clear and sharp.

(c) Incubate at 37° C. for 2 hours, examining at 30 minute intervals for the formation of precipitate at the juncture of antiserum and antigen. This is the *precipitin ring test.*

(d) Shake the tubes to thoroughly mix the antiserum and antigen, and store overnight in the refrigerator.

(e) Next day the precipitate will have settled out and can be read by gentle agitation of the tubes.

(f) It is essential that controls of antigen plus saline, and antiserum plus saline, be included, and these should show no precipitate.

The precipitin test may also be carried out in capillary tubes to conserve antiserum, and to utilize the relatively small quantities of antigen available in bacterial extracts as in the typing of streptococci.

Flocculation Tests. The so-called flocculation tests developed for the serological diagnosis of syphilis are related in a sense to the precipitin reaction, and the Kahn test is typical of these. The Kahn antigen is a lipid fraction of heart muscle soluble in alcohol but insoluble in acetone, and contains added cholesterol. When this antigen is mixed with saline in certain proportions floccules appear, and the antigen is titrated to determine the smallest amount of saline which, when added to 1.0 ml. of antigen, produces aggregates which completely disperse upon the addition of more saline. Syphilitic serum has the property of preventing this dispersion. The test is based on the mixture of constant amounts of serum with varying concentrations of antigen which are thoroughly mixed by shaking in a shaking machine, and the addition of saline. The test is set up according to the accompanying protocol and read immediately and again 10 minutes after the addition of saline. A positive reaction is indicated by the presence of floccules in the mixture.

PROTOCOL FOR THE KAHN FLOCCULATION TEST

Tube Number	Antigen (ml.)	Serum (ml.)	Serum:Antigen Ratio	Shake vigorously for 2 minutes	Saline (ml.)
1	0.05	0.15	3:1		0.5
2	0.025	0.15	6:1		0.5
3	0.0125	0.15	12:1		0.5

Agglutination. Bacteria, and other particulate antigens, aggregate in clumps in the presence of homologous antiserum, and are said to be agglutinated. The agglutination reaction is useful in the serological identification of bacteria, especially those of the group of enteric bacilli such as the Salmonella and dysentery bacilli. It is also the immunological reaction made use of, in conjunction with agglutinin absorption, in the antigenic analysis of bacteria.

The agglutination reaction differs from the precipitin reaction in that the antiserum rather than the antigen is diluted, and the antibody titer of the antiserum is expressed as the highest dilution in which a constant amount of bacterial antigen is agglutinated. Agglutinating antisera commonly show titers ranging from 1:1000 to 1:10,000, and occasionally may reach titers as high as 1:50,000 to 1:100,000 though this is rare. The agglutination of bacteria may be observed either microscopically or macroscopically; the former is generally used for typing purposes and the latter in the titration of antibody.

The Microscopic Agglutination Test. In the microscopic agglutination test either only a few, *e.g.*, two or three, dilutions of antiserum are used, or, more commonly, a single dilution is used which preliminary titration has indicated will produce a rapid and complete agglutination with minimal cross reactions. This last allows the use of a single rapid test for typing and certain diagnostic purposes. The test is carried out as follows:
 (1) Place a loopful of saline solution on a clean cover slip, and with the straight inoculating wire add a small amount of bacterial growth. The suspension should be faintly turbid and when this point is reached, the needle is flamed and then used to stir the drop to a homogeneous turbidity.
 (2) Place a loopful of diluted antiserum beside the drop of bacterial suspension. Traces of serum remaining on the loop are, of course, removed by flaming.
 (3) With the inoculating needle mix the two thoroughly. Note that this produces an additional dilution of the antiserum, and the final dilution is approximately twice the original dilution.
 (4) Mount the cover slip on a hollow ground slide with petroleum jelly as in making a hanging drop preparation. No special incubation is necessary, and in a few minutes to an hour at room temperature agglutination occurs.
 (5) The preparation may be examined under the low power objective for a curdled appearance, or under high power or oil immersion objectives for direct observation of the agglutinated bacteria. False clumping may occur and is distinguished from true agglutination in that in the latter all of the bacteria are gathered in large clumps with none about the edges of the drop, but false clumping occurs in small aggregates about foreign particles and around the edge of the drop.

Macroscopic Agglutination. The titration of agglutinin is carried out in a manner similar to the titration of precipitin except, as noted above, with varying amounts of serum in the presence of a constant amount of antigen.
 (1) PREPARATION OF ANTIGEN:
   (a) Inoculate an agar slant culture with the bacteria to be used as antigen, spreading the inoculum over the entire surface of the slant.
   (b) After 18 hours' incubation run about 2 ml. of saline solution on the agar slant culture. The saline may contain 0.5 per cent formalin if a formalinized antigen is to be used.
   (c) Rub up the bacterial growth with an inoculating needle or loop to give a uniform suspension of the bacteria in the saline.
   (d) With flamed forceps remove a small piece of cotton from the bottom (sterile) part of the cotton plug of the culture tube and drop it into the saline suspension.
   (e) With a sterile pipette push the piece of cotton to the bottom of the saline, taking care to leave the cotton over the pipette opening.
   (f) Draw up the bacterial suspension into the pipette through the cotton, which serves to filter out coarse particles, and add this suspension slowly to the proper

turbidity to whatever volume of saline solution is required for the titrations to be undertaken. (A single agar slant culture will make 100 to 300 ml. of antigen depending on the amount of bacterial growth and the density of the final antigen.) The turbidity of the final antigen should be such that, when diluted with an equal volume of saline, it will be lightly turbid in the test tubes used for the titration. The precise turbidity is dependent upon the kind of antigen and antibody and on the personal preference of the worker.

(2) PREPARATION OF SERUM DILUTIONS:

(a) Serum may be diluted in essentially the same manner as antigen is diluted in the precipitin test, and the dilution is carried out in 10 x 75 mm. test tubes used for the titration of agglutinin since the antigen is added directly to the diluted serum. A common procedure is to set up a series of saline blanks, the first containing 0.9 ml. and the remainder 0.5 ml. The first dilution is 1:10, made by adding 0.1 ml. of serum to the 0.9 ml. blank; the second and subsequent dilutions are made by transfer of 0.5 ml. portions of each preceding dilution. This gives dilutions of 2^n, *i.e.*, 1:10, 1:20, 1:40 . . . etc.

(b) The above method is the one of choice when only a very few titrations are to be run, but is laborious and time-consuming if there are many titrations. An alternative, more efficient procedure less subject to dilution error arising from carrying over excess serum in the pipette, since so few pipettes are used that a fresh one for each dilution is practical, is shown in the accompanying table.

PROTOCOL OF AGGLUTININ TITRATION

Serum Dilution			Agglutinin Titration				
Serum (ml.)	Saline (ml.)	Dilution	Tube Number	Saline (ml.)	Serum (ml.)	Antigen (ml.)	Final Dilution
A 0.1 of undiluted serum	4.9	1:50	1	0	0.50	0.5	1:100
			2	0.25	0.25	0.5	1:200
			3	0.40	0.10	0.5	1:500
B 0.5 of dilution A	4.5	1:500	4	0	0.50	0.5	1:1000
			5	0.25	0.25	0.5	1:2000
			6	0.40	0.10	0.5	1:5000
0	0		control	0.50	0	0.5	

In this method only one direct dilution is made for each three final serum dilutions, and the dilutions closely approximate 2^n.

(3) PROCEDURE OF THE TEST:

(a) To each of the series of tubes containing 0.5 ml. each of the several serum dilutions, add 0.5 ml. of antigen suspension and mix by gentle agitation. Note that this doubles the serum dilution, and the final dilutions are 1:20, 1:40, 1:80 . . . etc., when made by the first method; the final dilutions in the second method are given in the table.

(b) The method of incubation is variable, depending upon the particular system being titrated, and the following methods are common:

(i) Incubate at 37° C. or 55° C. for 2 hours, read and store overnight at room temperature or in the refrigerator, and read again.

(ii) Incubate at 37° C. overnight and read the following morning.

(c) The agglutinated bacteria form clumps of precipitate in the bottom of the tubes and are read in a cross light to give a Tyndall effect by gently agitating the tubes individually to swirl the agglutinated bacteria up into the supernatant. The character of the agglutinated bacteria differs in different circumstances; the H agglutination of Salmonella, for instance, is flocculent, while the O agglutination is finely granular. Some workers record degrees of ag-

glutination, from + or barely perceptible partial agglutination to ++++ of complete agglutination with a clear supernatant, while others simply record the presence or absence of agglutination with a single + sign. The agglutinin titer of the antiserum is the highest dilution in which definite agglutination occurs.

Complement Fixation. The complement-fixation test is based on the observation that complement, a heat-labile constituent of normal serum, combines with an antigen-antibody complex and is said to be fixed. Since this fixation produces no visible change, a hemolytic system, consisting of sheep erythrocytes and anti-sheep cell hemolysin, is added. If the complement is free, *i.e.*, has not been fixed, and inferentially the original union of antigen and antibody has not occurred, the erythrocyte-hemolysin complex combines with the complement, and visible lysis of the red cells occurs. Conversely, if the erythrocytes are not lysed, the test antigen-antibody system has combined and fixed the complement, leaving none for hemolysis. This complement-fixation test is most often applied in the serodiagnosis of syphilis, but has proved very useful with the rickettsiae. Bacterial antigens fix complement readily.

The following reagents are required:
(1) The antigen-antibody system to be tested, either component of which may be unknown.
(2) Anti-sheep erythrocyte hemolysin, prepared by the immunization of rabbits with sheep red cells.
(3) A 2 per cent (by volume of packed cells following centrifugation) suspension of washed sheep erythrocytes in saline.
(4) A source of complement, practically always fresh guinea pig serum.

Titration of Reagents. Since the test is necessarily quantitative with respect to the relative proportions of the various components, the activity of these must be titrated prior to the actual test. These preliminary titrations include:
(1) The titration of hemolysin which is carried out by varying the amount of hemolysin in the presence of constant amounts of sheep erythrocytes and complement, the last in amounts more than necessary for complete lysis. This titration is carried out as indicated in the accompanying protocol. The smallest amount, *i.e.*, highest dilution, of hemolysin required to bring about complete lysis is the *hemolytic unit*. In the final test 2 units in a volume of 0.5 ml. are used in each tube and on the basis of this titration the hemolysin is diluted to contain 4 units/ml.

PROTOCOL OF HEMOLYSIN TITRATION

Tube Number	Hemolysin Dilution (0.5 ml.)	Complement 1:10 Dilution (ml.)	2% Suspension Erythrocytes (ml.)	Saline (ml.)
1	1:1000	0.3	0.5	1.7
2	1:2000	0.3	0.5	1.7
3	1:3000	0.3	0.5	1.7
4	1:4000	0.3	0.5	1.7
5	1:5000	0.3	0.5	1.7
6	1:6000	0.3	0.5	1.7
7	1:8000	0.3	0.5	1.7
8	1:10,000	0.3	0.5	1.7
control (9)*	1:1000	none	0.5	2.0
control (10)*	none	0.3	0.5	2.2

* both controls must be negative

PROTOCOL OF COMPLEMENT TITRATION

Tube Number	1:30 Dilution Complement (ml.)	Saline (ml.)	Hemolysin 4 Units/ml. (ml.)	2% Suspension Erythrocytes (ml.)
1	0.10	1.9	0.5	0.5
2	0.15	1.9	0.5	0.5
3	0.20	1.8	0.5	0.5
4	0.25	1.8	0.5	0.5
5	0.30	1.7	0.5	0.5
6	0.35	1.7	0.5	0.5
7	0.40	1.6	0.5	0.5
8	0.45	1.6	0.5	0.5
9	0.50	1.5	0.5	0.5
control (10)*	0.00	2.5	0.0	0.5

* control must be negative

(2) The complement of fresh guinea pig serum is similarly titrated, using variable amounts of complement in combination with constant amounts of erythrocytes and hemolysin as shown in the accompanying protocol, and the unit of complement is defined. The "exact unit" is the smallest amount that gives complete hemolysis, and the next larger amount, *i.e.*, in the adjacent tube, is the "full unit." In the complement fixation test 2 full units in a volume of 1.0 ml. are used in each tube. The reason for the excess amount is that some complementary activity is lost during the time allowed for fixation, even if no fixation occurs, and the test is sufficiently sensitive even with this slight excess.

(3) Certain properties of the antigen are pertinent to the test and must also be determined by preliminary titration. These properties include anticomplementary activity, *i.e.*, inhibition of the action of complement, hemolytic activity, and binding power for complement. The titration of these is shown in the accompanying protocol. In general, the binding power of the antigen should be at least 10 times its anticomplementary action, and in the final test not more than one-third of the amount of antigen found to be anticomplementary may be used. It is self-evident that the antigen must not lyse red cells in the concentrations used in the test.

Procedure of the Test:

(1) Inactivate the test serum, *i.e.*, destroy any traces of complement in it, by heating to 56° C. for 15 to 20 minutes, and dilute 1:5, and distribute in three 15 x 100 mm. test tubes as indicated in the accompanying protocol. Bring the total volume in tubes 2 and 3 to 0.5 ml. by adding 0.25 and 0.375 ml. of saline respectively.

(2) Add 0.5 ml. of antigen, diluted to contain 20 complement-fixing units per ml. to each tube.

(3) Let the mixture stand at room temperature for 10 minutes.

(4) Add 1.0 ml. of complement, diluted to contain 2 full units per ml., to each tube.

(5) The mixture may be incubated in a water bath at 37° C. for 1 hour, or stored in the refrigerator overnight and warmed in the water bath for 10 to 15 minutes before proceeding with the test the following day. This time interval is allowed for fixation of the complement by the antigen-antibody complex, and the latter method is referred to as "ice box fixation."

(6) Add 0.5 ml. of hemolysin diluted to contain 4 hemolytic units per ml. to each tube.

(7) Add 0.5 ml. of a 2 per cent suspension of washed sheep erythrocytes to each tube.

(8) The appropriate controls of the serum, antigen, hemolytic system and erythrocyte suspension are indicated in the protocol, and *must be included.*

(9) Incubate in a water bath at 37° C. for 15 to 60 minutes.

(10) Read the hemolysis as complete (++++), partial (+++, ++, +, ±) or negative.

PROTOCOL OF ANTIGEN TITRATION

Property Tested	Tube Number	Antigen* (ml.)	Antiserum* 1:25 Dilution (ml.)	Complement 1:10 Dilution (ml.)	Saline (ml.)	37° C. for 1 hour or store in refrigerator overnight	Hemolysin 4 Units/ml. (ml.)	2% Suspension Erythrocytes (ml.)
Anticomplementary Activity	1	0.5	0	0.3	0.2		0.5	0.5
	2	0.4	0	0.3	0.3		0.5	0.5
	3	0.3	0	0.3	0.4		0.5	0.5
	4	0.2	0	0.3	0.5		0.5	0.5
	5	0.1	0	0.3	0.6		0.5	0.5
	6	0.05	0	0.3	0.65		0.5	0.5
Hemolytic Activity	7	0.5	0	0	1.0		0	0.5
	8	0.1	0	0	1.4		0	0.5
Complement-binding power	9	0.5	0.25	0.3	0.05		0.5	0.5
	10	0.25	0.25	0.3	0.2		0.5	0.5
	11	0.1	0.25	0.3	0.35		0.5	0.5
	12	0.075	0.25	0.3	0.375		0.5	0.5
	13	0.05	0.25	0.3	0.4		0.5	0.5
	14	0.025	0.25	0.3	0.425		0.5	0.5
Controls	15†	0	0.25	0.3	0.45		0.5	0.5
	16‡	0	0.25	0	1.75		0	0.5

* The amounts of antigen and antiserum given here are arbitrary and will vary with individual preparations

† This control should show complete hemolysis

‡ This control should show no hemolysis

PROTOCOL OF THE COMPLEMENT FIXATION TEST*

Tube Number	Serum Diluted 1:5 (ml.)	Antigen 20 Units/ml. (ml.)	Saline (ml.)		Complement 2 Units/ml. (ml.)		Hemolysin 4 Units/ml. (ml.)	2% Suspension Erythrocytes (ml.)
1	0.5	0.5	0	Let stand at room temperature for 10 minutes.	1.0	37° C. for 1 hour, or refrigerator overnight and 5-10 min. 37° C.	0.5	0.5
2	0.25	0.5	0.25		1.0		0.5	0.5
3	0.125	0.5	0.375		1.0		0.5	0.5
4 (SC)	0.5	0	0.5		1.0		0.5	0.5
5 (AC)	0	0.5	0.5		1.0		0.5	0.5
6 (HC)	0	0	1.0		1.0		0.5	0.5
7 (EC)	0	0	2.5		0		0	0.5

* Set up as the Kolmer modification of the Wassermann test

SC serum control—should show complete hemolysis
AC antigen control—should give complete hemolysis
HC hemolytic system control—should give complete hemolysis
EC erythrocyte control—should give no hemolysis

MORPHOLOGY, CELL STRUCTURE, GROWTH AND CHEMICAL COMPOSITION OF BACTERIA

Bacteria are widely distributed over the surface of the earth—in the soil, in the sea and in fresh water, and on the bodies of plants and animals. The true bacteria constitute only a portion, though a very large portion, of this vast microbial population, the remainder consisting of unicellular protozoa and fungi, including yeasts, molds and actinomycetes. This microbic population far exceeds, in point of numbers of individuals, that of all macroscopic plants and animals combined. So ubiquitous are the bacteria that there are few places to which man has access where they may not be found. They are present in the upper atmosphere and deep in the earth and sea. With the exception of certain obvious places such as the interior of active volcanoes, they may be said to occur universally over the surface of the earth. The skin of animals is covered with, and their gastro-intestinal tracts contain, tremendous numbers of these organisms. It is often stated that bacteria are absent from the tissues of healthy animals, but it appears that this is not always true.

As the discovery of this microbic world necessarily awaited the development of optical systems which made bacteria visible, so the structure of our knowledge of these organisms is built upon concepts derived from direct observation. Although the morphology of bacteria will not carry us far in their taxonomy, nor will it give us any insight into their physiological activities, nevertheless knowledge of the size and shape of these microorganisms, their internal and external structures, their processes of division and other characteristics determinable by direct observation, is the solid foundation upon which bacteriology rests.

Size. Different kinds of bacteria vary considerably in size. They are ordinarily measured in terms of microns (μ). The micron, a unit of the metric system, is 0.001 millimeter or roughly 1/25,000 of an inch. The average bacterium of rod shape measures about 2 μ in length and 0.5 μ in diameter. One large spherical bacterium has been described that measures about 2 μ in diameter; the most common microbe found in suppurative processes is a spherical bacterium about 0.8 μ in diameter. The largest bacteria belong, as a rule, to the group of spirally twisted or screw-shaped forms[1]; one of these[2] has been found to measure as much as 3.5 μ in diameter. Perhaps the largest pathogenic bacterium is the spirochete of relapsing fever, which may measure

---

[1] A bacillus (*B. bütschlii*), however, studied by Schaudinn (Arch. f. Protistenk., 1902, 1:306) measures from 50 to 60 μ in length and from 4 to 5 μ in width.

[2] *Spirillum colossum* (Centralbl. f. Bakt., 1902, Abt. II, 9:608).

up to 40 μ in length. One of the smallest of the well-known pathogenic forms is the so-called "influenza bacillus," which is about 0.5 μ by 0.2 μ.

Not only does the size of bacteria vary considerably from species to species but there is also great variability within a single species—much greater than one finds among many other forms of life. The bacillus of typhoid fever is found to range from 1 to 3 μ in length even when the descendants of a single cell, living under substantially identical conditions, are examined.

There is reason to suppose that still smaller organisms may exist. The infective agents of smallpox, infantile paralysis and a number of other diseases cannot be seen with the ordinary microscope and are so small that they will pass through filters that remove all known visible bacteria. These infective agents—known as the viruses or filterable viruses—are believed by many to be minute living organisms incapable of multiplication except in the presence of living host cells. It is open to question as to whether the viruses are related to ordinary bacteria. Measurements made by means of filtration through graded collodion membranes, which allow particles of known size to pass, indicate that the particles of certain viruses are as small as 0.01 μ in diameter.[3]

The mitochondria of living cells have been supposed by some investigators to be symbiotic bacteria, visible forms which are parasitic on the host cell as the viruses apparently are. However, microchemical and physical differences between bacteria and such structures render such a supposition unlikely.[4]

Morphology. For practical purposes the morphology of bacteria is best considered under two heads: the morphology of individual cells and groups of cells, or microscopic morphology; and the morphology of large aggregates of cells in bacterial colonies, macroscopic or colonial morphology. The individual cells differ in size, shape and other structural details, features which can be determined only by the use of high magnification. Colonies or masses of cells that develop on the surface of solid media often present peculiarities of form, color, consistency and the like which are apparent upon examination with the naked eye or low power lenses. As will appear, there is a relation between colonial and cellular morphology. Analogous differences may be observed between masses of larger objects which are associated with the appearance of the single objects. A grove of oak trees viewed from a distance too great to permit identification of the individual trees will still appear unlike a grove of pine trees.

Shape. With respect to shape, bacteria exist in three principal types: the spherical or coccus form; the rod-shaped form known as the bacterium or bacillus; and the spiral forms, the various subtypes of which are designated as vibrio, spirillum and spirochete.

Coccus. The cocci may be subdivided on the basis of the positions which the individual cells tend to take with respect to one another. Such groupings are primarily a result of the planes in which cell division takes place and the behavior of the daughter cells after division is complete. Those organisms which separate completely after cell division and appear singly and scattered at random over the microscopic field are designated as *micrococci*. In some

[3] The millimicron (mμ) is often used as the unit for such measurements. One μ is equal to 1000 mμ.

[4] Cowdry and Olitsky: Jour. Exp. Med., 1922, 36:521.

species, however, there is a tendency for the individual cells to remain in pairs, and one may observe such pairs intermingled with individual cells. These organisms are called *diplococci*. When this pairing tendency is marked as, for example, in the gonococcus, the individual cells may not be perfectly spherical but exhibit a coffee bean shape with the concave sides facing each other. Often coccus forms tend to remain together in sheets or irregular clusters resembling bunches of grapes. Those which assume this grouping are termed *staphylococci*. Division in two or three planes is, of course, necessary to the formation of such groups. Other coccus forms divide in only one plane but tend to remain together, the result being a chain of cocci. Organisms forming such chains are
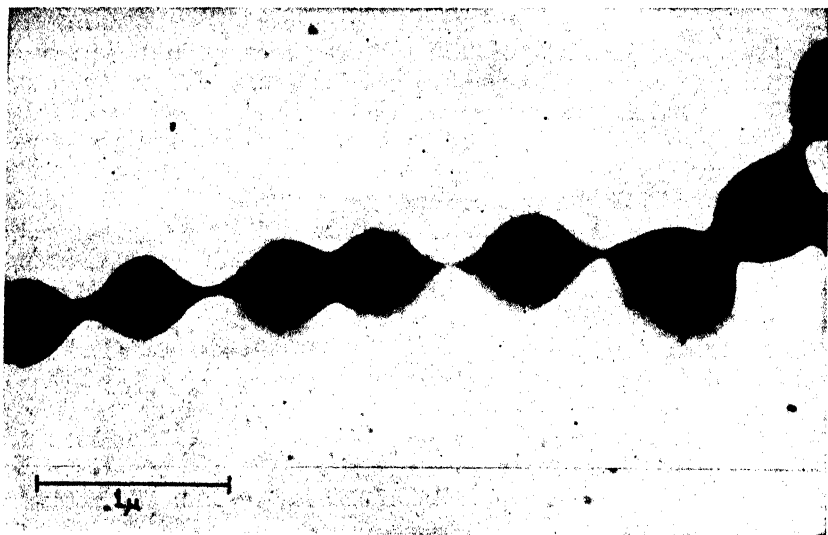
Fig. 2. Electron micrograph of *Streptococcus pyogenes*. Note the manner in which the individual cells remain attached to one another following fission, and the tendency of the chains to be made up of attached pairs of cells. (SAB No. 71.)

called *streptococci*. Still other cocci divide in three planes and remain together to form cubical packets of eight cells. These organisms are the *sarcinae*. Not all the cells in a given microscopic field will appear in characteristic groups. Many organisms may be found singly, presumably those which have broken away from the parent group. Chain formation by streptococci is influenced by the medium upon which the organisms have been grown. The acid-producing streptococci of milk, for example, show long chains in milk culture but when grown in nutrient broth may show only a few short chains. In smears of pus or similar material, however, the coccus forms are usually found in their characteristic groupings. Of the coccus forms having definite grouping, the staphylococci are the most consistently characteristic under the microscope.

Bacillus. The morphology of individual rod-shaped bacteria differs considerably from species to species. Not only is there variation in size but the shape of the individual cells differs. Some bacilli have sides more or less parallel with one another but rounded ends. Others have quite square ends which

appear to have been cut off sharply. Still others assume a highly elongated but oval shape. These last, when short and thick, are often called *coccobacilli*. A given shape is relatively constant within a species although the length-width ratio may vary considerably. The groupings which the cells may assume are a result of post-fission movements, for the plane of division varies rarely if at all. The tendency to remain attached, end to end, resulting in the formation of a chain of *streptobacilli,* is more or less constant for some species but is observed only occasionally in many species. Other groupings of these cells result from the movements of the bacilli after division is complete. Some tend to slide together side by side, a movement known as *slipping,* which results in the formation of palisade-like groups of cells. Still others, in a movement
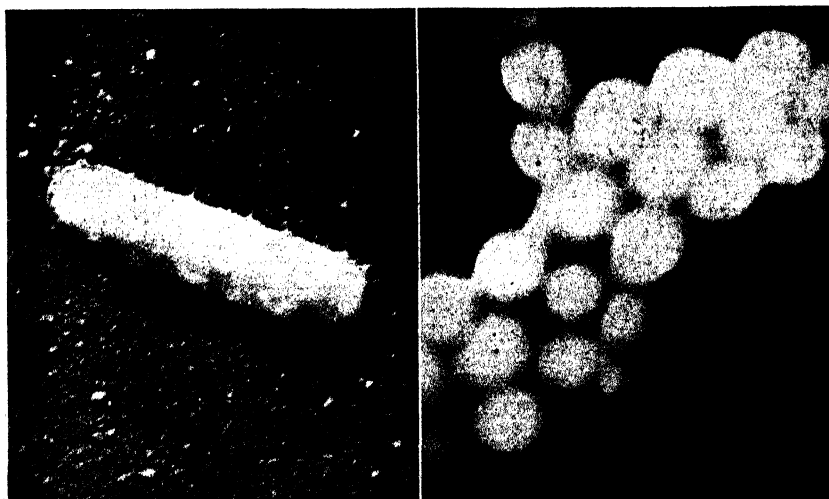
Fig. 3. Electron micrographs of gold-shadow-cast preparations of *Bacillus subtilis* and *Staphylococcus aureus*. The shadow-cast preparation illustrates particularly well the occurrence of staphylococci in clusters of attached cells. The cap at the ends of the bacillus and a single flagellum are shown. (Lilly Research Laboratories.)

known as *snapping,* bend sharply at the point of division to an acute angle and present a V-shaped appearance. When this type of movement is associated with a tendency for the bacilli to remain attached end to end, chains of organisms resembling a split rail fence may result. Such snapping often results in breakage of a chain of organisms, the parts of which continue to grow in the new direction. The groupings assumed by bacilli are, however, not as characteristic as those of the cocci.

   The Spiral Forms. The third principal type of bacterial form commonly observed is that of the spiral or screw-shaped forms. There are a variety of these which may be distinguished morphologically, but at the moment only three main subtypes may be considered. The first of these is the *vibrio* or curved rod. Smears of such organisms often show chains which, since the organisms curve in alternate directions, suggest a spiral organism. It is not difficult, however, to distinguish between such chains and the truly spiral

organisms. A spiral organism which is rigid, or relatively so, is called a *spirillum*, while similar organisms which are flexible are termed *spirochetes*. Further distinctions, such as the presence or absence of an undulating membrane, etc., which are made use of in formal taxonomic schemes, need not concern us here (see p. 728).

Involution Forms. Although the vast majority of bacteria exhibit a marked constancy of form during the early stages of the growth of a culture, in older cultures aberrant forms such as over-sized cocci, Y-shaped bacillary forms and forms apparently containing considerable quantities of granular material may be found. These are generally regarded as *involution* or *degenerative* forms which are dead or dying and represent varying stages in the dis-
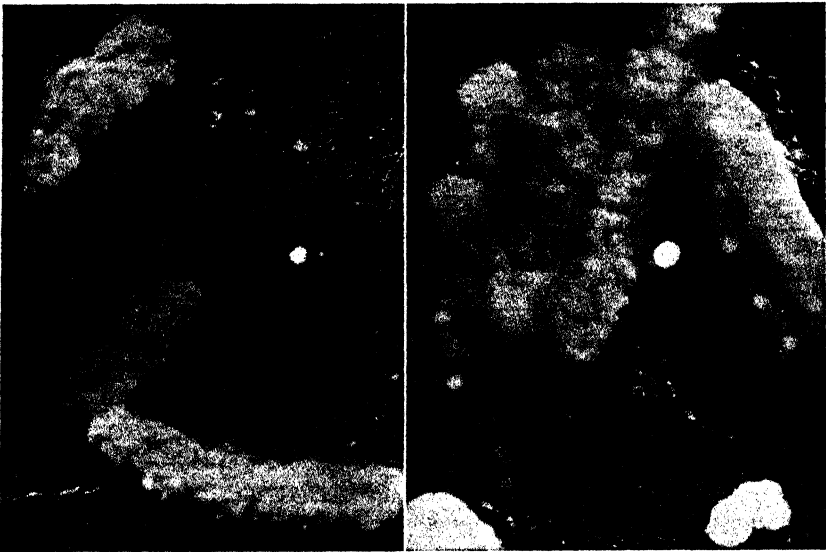
Fig. 4. Electron micrographs of gold-shadow-cast *Bacterium coli* from old cultures. One intact cell is present, and the remainder show the granulation of dead and disintegrating cells. (Lilly Research Laboratories.)

solution of the cell structure. Some workers, however, attach significance to such forms and consider them as indicative of the existence of complex life cycles. Such irregular bizarre forms may often be produced at will by cultivation of bacteria under somewhat adverse conditions of temperature, salt concentration and the like. Morphological variations and their implications are discussed at greater length elsewhere (Chap. 6).

THE STRUCTURE OF BACTERIAL CELLS[5]

The structure of bacterial cells, particularly their finer structure, has been of considerable interest since these organisms were first studied. They are so small, however, that the limitations imposed by the optical system employed become of considerable importance.

[5] The cytology of bacterial cells is discussed at length by Lewis: Bact. Rev., 1941, 5:181; and by Knaysi: *Elements of Bacterial Cytology*. Comstock Publishing Co., Ithaca. 1944.

The Compound Microscope. With the ordinary microscope objects are viewed by transmitted light and therefore must be sufficiently large to cast a shadow. This size is approximately half the wave length of the light used. The highest numerical aperture that is practical in the objective is 1.4. Such a lens, when used with monochromatic light with a wave length of 5460 Å or 546 mμ, will resolve a particle having a diameter of 0.2 μ. Objects somewhat smaller than this may be seen in that they are visible but are not resolved and neither size nor shape can be determined. Since many bacteria are no wider than 0.2 to 0.5 μ the hope of observing intracellular structures is a faint one. Some workers, Barnard[6] in particular, have attempted to extend the limits of resolution through the use of ultraviolet light and quartz lenses coupled with "seeing" by means of a photographic plate, but such efforts have not added greatly to knowledge.

The Darkfield or Ultramicroscope. In the instrument known as the darkfield (*Dunkelfeld*) or ultramicroscope, reflected rather than transmitted light is used, objects appearing brilliantly lighted against a black background. The usual microscope may be used as a darkfield by attaching a darkfield condenser in place of the Abbé substage condenser. The principle is the Tyndall effect, perhaps most commonly observed in the appearance of particles of dust in a shaft of sunlight in a darkened room. The limit of resolution is not increased, however, and while exceedingly small objects are visible, they appear only as brilliant points of light. The use of this type of microscope has not contributed materially to knowledge of the structure of the bacterial cell except with regard to the mode of action of flagella. It is, however, very generally used for the detection and observation of very slender microorganisms, especially spirochetes as in exudate from a syphilitic chancre or leptospira in the blood from cases of leptospirosis.

The Electron Microscope.[7] The recently developed instrument known as the electron microscope operates on a different principle from the optical microscopes in that a stream of electrons is used rather than a beam of light. The electron stream is focused by means of magnetic "lenses," and the interposed object intercepts the stream to cast a "shadow" which is recorded on a photographic plate to give an electron micrograph. Untreated bacteria cast shadows in the electron beam, or the preparation may be "metal shadowed" by the deposition of gold, chromium, etc., at an angle so that the resulting micrograph has a three-dimensional appearance. Remarkable resolution is possible because of the minute size of the electron and sharp photographs may be obtained at magnifications of 30,000 diameters or more. Its application is somewhat limited in that the object must be prepared as a dry film on collodion and observed in a high vacuum; in consequence, living organisms cannot be studied. Application of the electron microscope to biological problems is still in its infancy but has already contributed considerably to knowledge of bacterial cell structure.

Certain structures of bacterial cells are, however, of sufficient magnitude to be seen and studied with the optical microscope. The most obvious of these are structures external to the cell—capsules and flagella.

[6] Barnard: Lancet, 1925, ii:117.

[7] See Burton and Kohl: *The Electron Microscope: Its Fundamental Principles and Applications.* 2nd ed. Reinhold Publishing Corp., New York. 1946.

Capsules. Many bacteria, perhaps potentially all, form a capsule, which apparently originates from the outer layer of the cell membrane; in stained preparations it can sometimes be seen surrounding the cell like a halo. The capsules of bacteria are often one or two times as thick as the diameter of the cell itself and may appear to extend continuously over a chain of organisms or completely surround paired organisms such as diplococci. Cultures of capsulated bacteria are usually slimy and the growth is sticky and viscous. The capsular material is generally polysaccharide in nature, though some bacterial slimes may contain mucin-like proteins. Some of these forms may be a source of considerable annoyance in the sugar refining industries. The presence of a capsule is also associated with the virulence of a variety of pathogenic bacteria. Pneumococci, for example, which have no capsule are relatively avirulent but when capsulated are highly virulent. Anthrax bacilli are almost always found to be capsulated when observed in preparations made from ani-
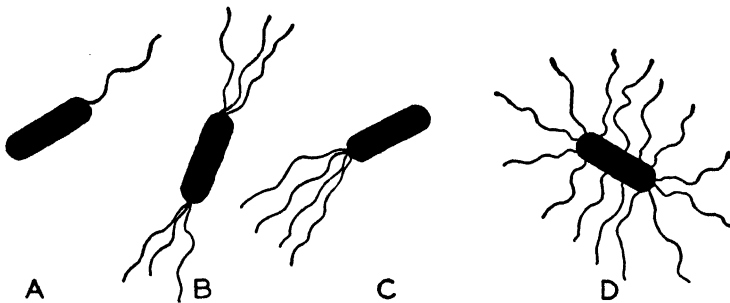
Fig. 5. Position of flagella on the bacterial cell. *A,* monotrichous; *B,* amphitrichous; *C,* lophotrichous; *D,* peritrichous.

mal tissues. The capsule appears to function as a bacterial defense against the activity of phagocytic cells of the body. The sheaths of some of the filamentous bacteria may possibly be a capsule in somewhat different form. The colorless sulfur bacteria which derive their energy through the oxidation of hydrogen sulfide to elementary sulfur deposit sulfur granules in their sheaths. Likewise the iron bacteria which oxidize iron from the ferrous to the ferric state deposit ferric hydroxide in the sheaths surrounding them.

Flagella. Many kinds of bacteria are observed to be motile under the conditions in which bacteria are usually studied. Some of those forms in which motion has never been observed may possibly possess the power of locomotion under certain unusual conditions. Independent bacterial motion is a true movement of translation, and is to be distinguished from the oscillating or quivering movement (brownian movement) exhibited by all very minute particles suspended in water or other fluids. Many bacteria are found to be motile when they are examined after removal from certain culture media, but non-motile if they have been grown on other substances. The colon bacillus, for example, is motile when picked from young colonies on gelatin or agar but is frequently non-motile when taken from broth. The rate at which a bacterium moves has been approximately measured. The typhoid bacillus may

travel a distance of 4 mm., or about 2000 times its own length, in one hour; the cholera vibrio may attain a speed of 18 cm. per hour for short distances.

This power of locomotion depends upon the possession of flagella, long, fragile, filamentous, coiled appendages. These organs are contractile and their propelling action is due, not to a lashing motion as once thought, but to a rhythmic contraction which moves helicoidally over the surface, (the action being that of a screw rather than an oar).[8] Flagella are very slender, 20 to 50 mμ in diameter, and their demonstration with the optical microscope commonly requires staining methods, such as silver impregnation or mordanting, by which staining material is deposited upon them to make them thicker than they actually are. It has been reported, however, that flagella may be observed in the living unstained state in some bacteria, such as the very large spirilla. The origin of flagella has been a matter of some interest and it has been generally
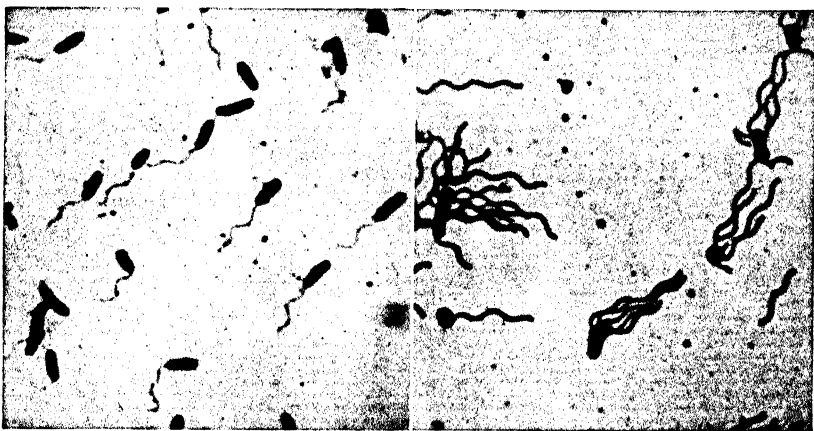
Fig. 6. Flagella of bacteria in stained smears examined with the optical microscope. Single polar flagella of a monotrichous bacterium (*Vibrio cholerae*) on the left, and peritrichous flagella (*Salmonella typhi-murium*) on the right; × 2000 (Kral).

assumed that they are extensions of the cell wall or protoplast.[9] Knaysi[10] has shown, by means of electron micrographs, that they originate in the protoplasm of the cell, and the more recent evidence of van Iterson and Robinow, illustrated in Fig. 7, indicates that their origin is in small spherical bodies lying between the cell wall and cytoplasmic membrane. Whether these correspond to the blepharoplasts of flagellated protozoa is not yet clear. It may be noted that the suggestion of Pijper[11] that flagella are capsular polysaccharide twisted off in threads by the motion of the cells, and therefore artifacts, has no basis in substantial evidence.

Bacteria differ from one another with respect to the position of the flagella on the cell body. The *monotrichous* organisms have only a single flagellum at one end; the *amphitrichous* bacteria have either a single flagellum or a tuft

[8] Pijper: Jour. Path. Bact., 1938, 47:1.
[9] See the review by Knaysi: Bot. Rev., 1938, 4:86, 99.
[10] Knaysi: Science, 1942, 95:406.
[11] Pijper: Jour. Biol. Photo. Assn., 1947, 16:3.

of flagella at each end; the *lophotrichous* bacteria have a tuft of flagella at one end; and the *peritrichous* have flagella projecting from the whole body of the cell, the sides as well as the ends. Bacteria having no flagella are termed *atrichous*.
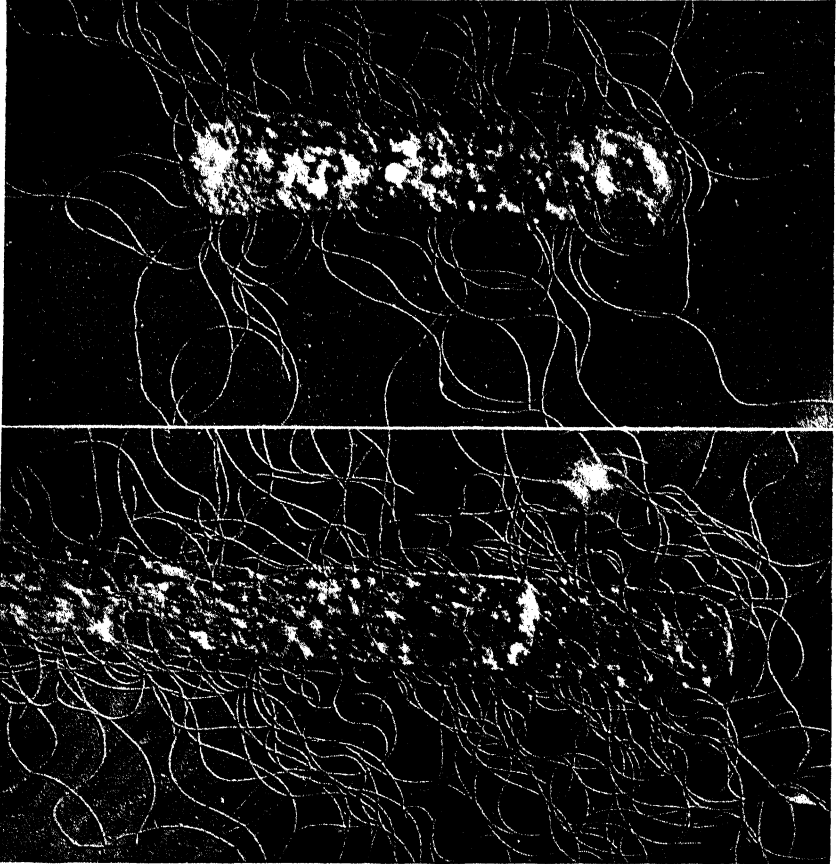
Fig. 7. Electron micrographs of shadow-cast preparations showing the flagella of *Proteus vulgaris*. The bacteria were grown to the swarming stage on agar, refrigerated at 5° C. for 16 to 20 hours, floated off in 5 per cent formalin and washed twice before mounting on collodion. The preparations are very transparent and flatten completely so that certain of the internal structures are demonstrable by shadowing. The origin of the flagella in small, spherical bodies about 100 mμ in diameter and remarkably uniform in size, situated between the cell wall and cytoplasmic membrane, is clearly shown. (From electron micrographs by Woutera van Iterson and C. F. Robinow. A full account of this work will appear in Biochimica et Biophysica Acta.)

The number of flagella on the peritrichous bacteria varies considerably and even closely allied species may differ from one another in this respect. The typhoid bacillus, for example, has, as a rule, more flagella (ten to twelve) than the colon bacillus (two to six). The majority of actively motile bacteria belong either to the bacilli or spirilla; very few cocci are motile under ordinary

conditions. Within a given bacterial species different degrees of motility may be found. Some strains within a species may consist practically entirely of motile cells, while other strains may show no motility in hanging drop preparations nor may flagella be found in stained smears. Further, the motility of some strains is affected by the temperature of incubation while that of others is not; some strains may be motile when grown at 22° C. but not when grown at 37° C. Motility, therefore, is not a character that can be used for exact delimitation of species or varieties of bacteria. Standard cultural characters and biochemical reactions do not appear to be correlated with the presence or absence of motility.

Fig. 8. Spore formation by bacteria. Left, the anthrax bacillus which forms central spores which do not distend the wall of the vegetative cell; the tendency of the bacilli to occur in chains is also characteristic. Center, *Clostridium botulinum* type B, which shows the typical clostridial form with terminal spore of greater diameter than the vegetative cell; note the free spores separated from disintegrated vegetative cells. Right, *Clostridium sporogenes,* showing a subterminal clostridial spore. These preparations are all stained with a single stain in the usual way; the vegetative cells take up the dye but the spores remain unstained. Fuchsin; × 1050.

Spores.[12] Some of the bacilli possess the ability to form resistant structures known as spores. Such spores, spherical or oval in shape, show a relatively high resistance to all sorts of injurious influences, including high temperatures, germicidal chemicals, etc. Furthermore, they stain with great difficulty. Usually heat in combination with a mordant is necessary to drive the dye into them.

An assembling or concentration of nuclear material (*i.e.,* material staining deeply with basic dyes) precedes spore formation in some cases and constitutes the spore primordium. Cytological studies suggest that in some bacteria a granule forms which enlarges to become the spore, in others there is an aggregation of granules, and in still others no granules are formed and what appears to be a condensation of the protoplasm occurs. The highly refractive character of the unstained spore and its reduced water content probably are connected with the concentrated character of the spore substance. Such spores

[12] See the review by Knaysi: Bact. Rev., 1948, *12*:19.

may be formed in any part of the cell, the position generally being relatively constant within a species. The spore is spoken of as *terminal* if formed at one end of the cell, *central* if formed in the center of the cell, and *subterminal* if formed half way between the center and the end of the vegetative cell. In some cases the spore does not exceed the diameter of the parent cell, as in the anthrax bacillus; in others where its diameter is greater than that of the vegetative cell it causes a bulging out of the wall of the cell at the point where it lies. If such spores are terminal, a drum-stick appearance may result, as in the tetanus bacillus; or if it is central, the vegetative cell becomes spindle-shaped. In simple stains the spore appears as an unstained round or oval body lying within the stained protoplasm of the vegetative cell. When the spore is fully developed, the vegetative cell disintegrates and disappears.
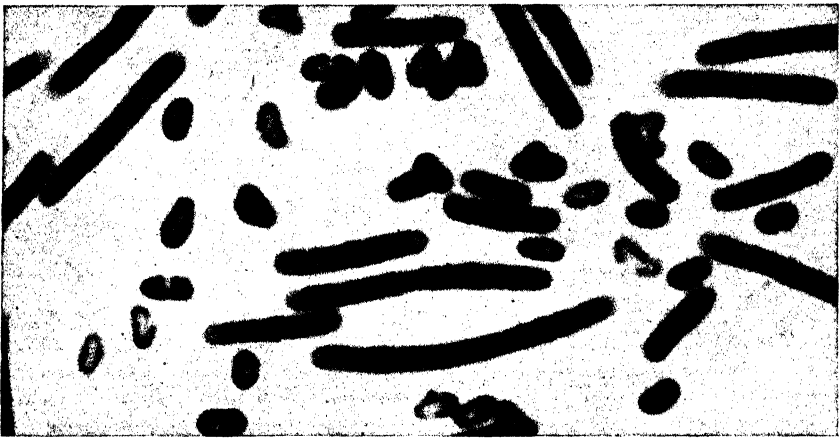
Fig. 9. Recently germinated vegetative cells of *Bacillus mesentericus*, showing discarded spore cases. Note the cell boundaries in the long filaments made up of cells attached end to end. The deeply staining areas are chromatinic bodies (see Fig. 14). Osmium tetraoxide-hydrochloric acid fixation, Giemsa; × 4000. (Robinow.)

The conditions under which bacteria form spores vary with the nature of the organism. The anthrax bacillus, an aerobe, forms spores only when in contact with free oxygen, a fact that has practical bearing on the disposal of bodies of cattle dead of the disease. The tetanus bacillus, on the other hand, and the anaerobes in general, form spores in the entire absence of oxygen. A suitable temperature is essential to the formation of spores. The anthrax bacillus forms spores most abundantly at 30° C. to 32° C. and will not produce them at a temperature of 12° C. Lack of food is apparently not an adequate stimulus to spore formation. In all cases a period of uninterrupted vegetative multiplication precedes the appearance of spores, and the conditions necessary for their production seem to arrive simultaneously for most of the cells in a culture. It has been suggested that diminution in the water content of the bacterial cell, leading to a shrinking of the colloids, is the main factor in bringing about spore formation. Friedman and Henry[13] have found that the percentage of bound water is relatively high in spores as compared with

[13] Friedman and Henry: Jour. Bact., 1938, 36:99.

vegetative cells, and suggest that this is assocated with their heat resistance. In any case, spores are not formed under adverse conditions, as it might be thought, but in circumstances favorable to growth.

When spores are brought under conditions favorable for growth they *germinate* and become vegetative cells which multiply in the usual manner. The rate of germination has been found to be logarithmic[14] but delayed germination occurs with some frequency and in some cases appears to be due to the presence of inhibiting substances, such as oleic and linoleic acids, in the culture medium rather than a delayed response to a favorable environment.[15] As it undergoes germination the spore shows a change in its refractive properties, probably due to the imbibition of water. The entire spore wall may
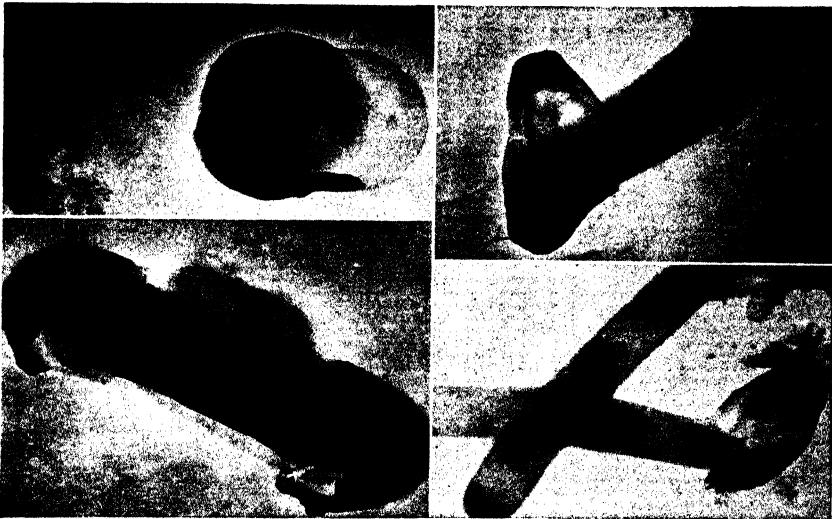
Fig. 10. Electron micrographs illustrating the process of germination of spores of *Bacillus mycoides*. In the upper left the vegetative cell is just beginning to emerge from the spore case. In the upper right the vegetative cell is growing out of the side of the spore case, and has broken the case in two parts in the lower left. In the lower right germination is complete. The darker areas in the vegetative cells are regarded by some as nucleus-like in nature. (Knaysi, SAB Nos. 203, 204, 205, 206.)

become thin and stretch and the spore assume a bacillary form, or a vegetative cell may burst through the spore membrane, the empty case being cast off as a hull. The anthrax bacillus grows out of one side of the spore wall; the closely related hay bacillus grows out of opposite sides simultaneously. Other forms of bacteria exhibit intermediate methods of germination. Also, irregularities may occur in the development of spores of the same species. The process of development of the vegetative cell and its escape from the spore case is particularly well illustrated in the electron micrographs in Fig. 10, taken by Knaysi who has studied the process of sporulation in detail.[16]

[14] Wynne and Foster: Jour. Bact. 1948, 55:69.

[15] See the general discussion of studies on this point by Foster and Wynne: Jour. Bact., 1948, 55:623.

[16] Knaysi: Jour. Bact., 1945, 49:473, 617; *ibid.*, 1946, 51:187; *ibid.*, 1947, 53:525.

Spore formation is not very common among bacteria. It has been definitely observed only in the rod forms; cocci are not known to sporulate, and sporulating spirilla, if they exist, are rare. The majority of the obligate anaerobes produce spores, as do some of the aerobic bacilli. The spore-forming bacteria of known pathogenicity for man are few, a fortunate circumstance that materially facilitates and simplifies disinfection and the treatment of infectious disease. Physiologically, the spore is usually considered as a resting stage, serving to tide the species over unfavorable periods. From this point of view, the spore stage is analogous to the periods of hibernation or estivation among higher
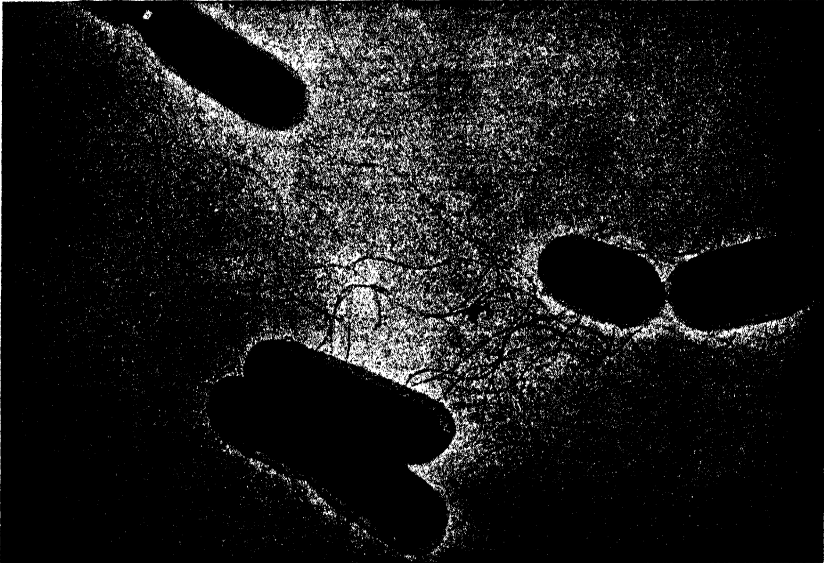
Fig. 11. Electron micrograph of *Clostridium tetani* from 24 hour culture. The protoplasm is homogeneous at this stage and the clear cell walls are apparent. Peritrichous flagella are shown also, and the bacilli are in various stages of cell division. Compare the latter with Fig. 15. (Mudd and Anderson, SAB No. 61.)

forms of life and the living matter of the spore may remain dormant for years or even decades. It does represent a definite differentiation of the vegetative cell as indicated by the occurrence of antigens in the spore that differ from those of the vegetative cell as well as antigens shared by the two forms.[17] Spore formation is not a form of multiplication, for each vegetative cell forms only a single spore and each spore germinates into a single vegetative cell. Obviously no increase in numbers is possible by this process.

The Finer Structure of the Bacterial Cell. Knowledge of the intracellular structures of bacteria is strictly limited, as pointed out above, by limits in the resolving powers of the microscope. Three questions have been of interest, namely: the existence and nature of the cell membrane; the nature of the cell substance or entoplasm; and, finally, the question of the existence of a nucleus.

[17] See Doak and Lamanna: Jour. Bact., 1948, 55:369.

The cell membrane of bacteria is not demonstrable as a structurally differentiated envelope, as that of many plant cells is, by immersion of the cells in hypertonic solutions. Robinow has found that treatment with boiling dilute sodium hydroxide results in a shrinkage of the protoplasm away from the cell wall, allowing its direct demonstration by staining with crystal violet. Such rigorous treatment may, of course, result in artifacts but a cell wall appears clearly in electron micrographs as shown in Fig. 11, and studies by Mudd and his associates[18] with the electron microscope have shown that it is a definite morphological structure from which the cytoplasm may shrink away and which is sufficiently solid to show jagged lines of fracture when broken by sonic vibrations. It would appear to have considerable rigidity, particularly among the bacilli, in order to maintain shapes other than spherical. Experi-
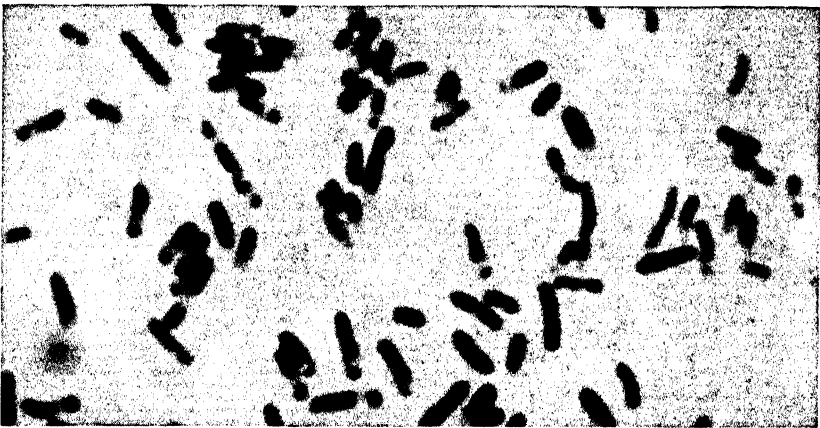
Fig. 12. Irregular and bipolar staining of the plague bacillus. Note the heavily stained areas and metachromatic granules. Fixed in methyl alcohol and stained with methylene blue; × 2400.

ments on the growth of the colon bacillus in media in which the surface tension had been reduced by the addition of sodium oleate resulted in the production of long, filamentous forms, much longer than the usual rods. Such experiments have been interpreted as indicating that this organism is essentially tubular, the sides having sufficient rigidity to withstand the reduced surface tension but the length increasing because of a less rigid structure at the ends.

Knaysi[19] has found that the cell wall of *Bacillus cereus* and *Bacillus megatherium* is made up of lipid and protein in stable combination and is much more resistant to autolysis than the cytoplasm. It stains with dyes of the Sudan series, and he has found that the inner surface is jagged and irregular, tending to divide the cell into compartments. It is possibly this last that results in the lack of a sharply defined interface between the entoplasm and the outer cell wall in the usual preparations. The precise nature of the bacterial cell membrane is of particular interest in connection with the problems of permeability.

[18] Mudd *et al.*: Jour. Bact. 1941, *41*:415; *ibid.*, 1941, 42:251.
[19] Knaysi: Jour. Bact., 1946, *51*:113.

The nature of the cell substance is not well known. It is, of course, protoplasm, as the ancillary evidence of chemical composition indicates. The cells of actively growing cultures appear to be homogeneous both in the unstained state and when stained by various dyes. As the culture becomes older, however, the cells of many organisms may be found to contain granular material which is somewhat more refractive than the surrounding protoplasm in the unstained state, and which takes up certain dyes with greater avidity than the remainder of the cell. These granules are called *metachromatic granules* or, after the workers who first described them, Babes-Ernst granules. They are sometimes scattered through the cell substance, and sometimes massed at either end, where they constitute the "polar granules" observed in the plague bacillus, the glanders bacillus and certain other bacteria. In certain species

Fig. 13. Metachromatic granules and bipolar staining in the diphtheria bacillus. Note the differences between these organisms and the plague bacilli. Methylene blue; × 1975.

the metachromatic granules are particularly easy to demonstrate, and their abundance may even constitute a character of some differential value. The nature of these granules has been the subject of microchemical investigations, but the results are somewhat vague. Some are lipid in nature and others consist of polysaccharide material which gives a color with iodine reagents and has been designated *granulose*. The greater proportion of these granules, however, appear to consist of nucleoprotein and take up the nuclear stains with great avidity. This nucleoprotein has been termed *volutin*, and granules of this and other material are generally spoken of as "reserve material"—a term which, because of its implications, could hardly have been less wisely chosen.

The apparent lack of a discrete nucleus in the cells of the true bacteria has been a source of much speculation. Granular materials such as volutin and metachromatic granules which are characterized by staining deeply with basic dyes are sometimes referred to as "nucleoids" but apparently have no nuclear function. The seeming homogeneity of the cell substance, both in the stained and unstained states, has been interpreted by some to indicate that these cells

consist entirely of cytoplasm and have no nucleus, and by others to mean that these cells are all nucleus and have no cytoplasm.

There is no doubt, however, that the bacterial cell contains relatively enormous amounts of nucleoprotein and nucleic acids demonstrable by micro-chemical methods such as the Fuelgen reaction, and the content of purine and pyrimidine nitrogen found by analysis is very high. It is clear that nuclear material is present in more than adequate amounts, and it has been customary to assume that the bacterial cell contains the chemical equivalent of a nucleus even though no morphologically differentiable structure could be demonstrated. More recently Robinow[20] has shown that the very faintly discernible areas apparent in bacteria stained with polychrome stains may be clearly shown by fixation with osmium tetraoxide, differentiation in hot hydrochloric acid, and staining with Giemsa. He has called these chromatinic bodies, and their normal occurrence has received strong support from the studies of Hillier, Mudd and Smith[21] who have found them in electron micrographs of preparations receiving no treatment other than drying. These chromatinic bodies are illustrated in both light and electron micrographs in Fig. 14. They show a strong morphological resemblance to nuclei, stain like nuclei with Giemsa, and appear to divide in the growing cell just prior to fission. Whether they represent true nuclei, analogous to those of other cells, remains to be fully established, but the implication that they are seems very strong.

COLONIAL MORPHOLOGY

A single bacterial cell or group of cells, when planted on a semisolid medium and allowed to develop under suitable conditions of moisture, temperature and air supply, will, in a few hours or days, develop a "colony" so large that it can be plainly seen with the naked eye. In many instances such masses of cells, particularly when the growth occurs on certain culture media, possess salient peculiarities which are characteristic of the species. Growths upon nutrient gelatin are especially characteristic, but upon nutrient agar the morphology of bacterial colonies is less distinctive. Colonies on potato and other solid food substances are, as a rule, still less characteristic. As was pointed out previously, colonial differences may often be accentuated by growth on media containing dyes and other compounds. Diphtheria bacilli, for example, form highly characteristic colonies on media containing potassium tellurite. The character of colonies is also somewhat affected by the density and viscosity of the culture medium and by the physical conditions under which the organisms develop. In general, however, the colony characteristics of a bacterial species are distinctive and are an aid in the isolation and identification of the organism. These characteristics include the size of the colony, its form—whether raised, flat, etc.—and the shape of its edges; its consistency and texture; its surface—whether roughened or smooth; its color; and other similar peculiarities.

These colony characteristics in many cases have their roots in the mor-

[20] Robinow: Jour. Hyg., 1944, *43*:413.
[21] Hillier, Mudd and Smith: Jour. Bact., in press; personal communication from Dr. Mudd.
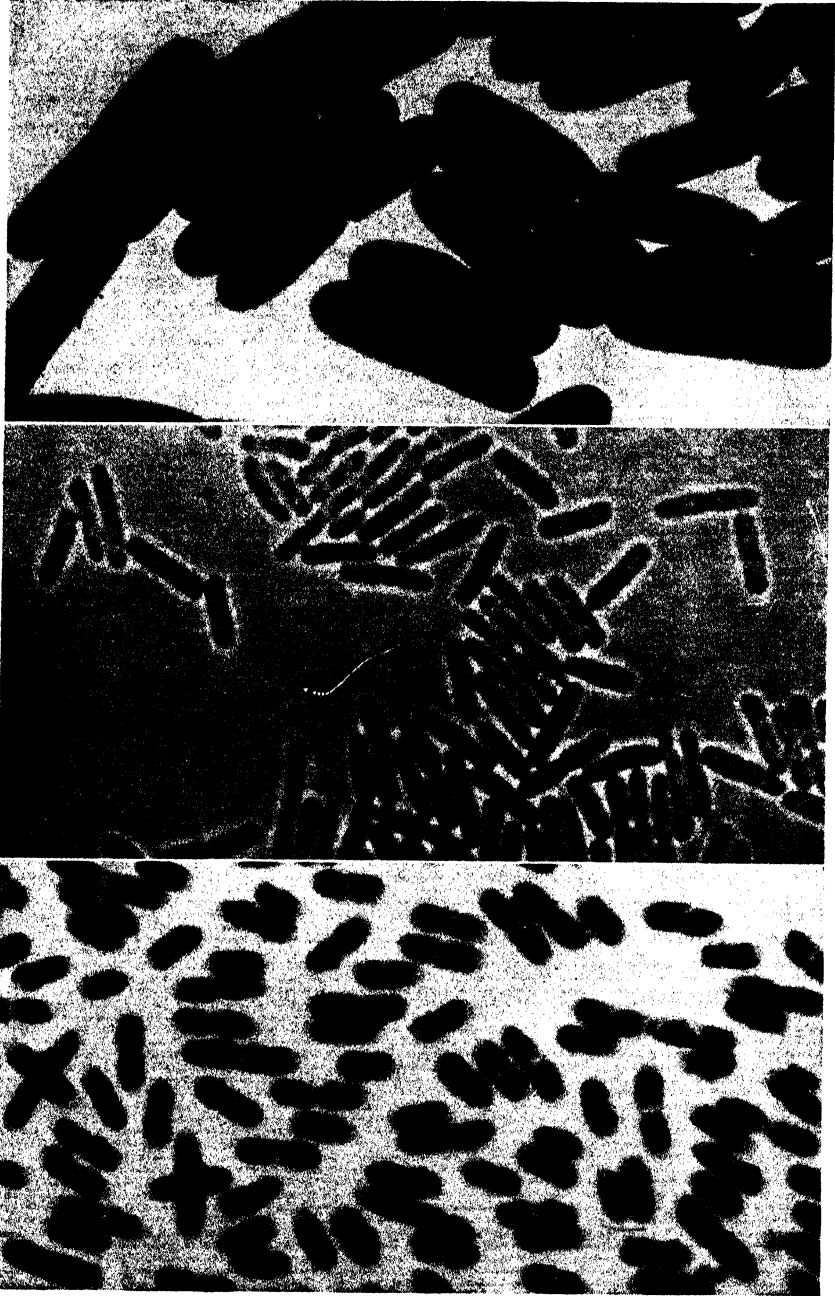
Fig. 14. The chromatinic bodies of *Bacterium coli* which are interpreted as representing the bacterial nuclear apparatus. The upper section of the plate is an electron micrograph of an unfixed preparation from a 3 hour culture of *Bact. coli*. The light areas within the bacterial protoplasm correspond with those faintly discernible with the light microscope and clearly discernible after fixation with osmium tetraoxide vapor. (Hillier, Mudd and Smith,

phology of the individual cells. The movements of cells after fission and the arrangements that result determine, to a considerable extent, the texture of a colony and the shape of its edges. The long coiled filaments of the anthrax bacillus give the colony of this organism its characteristic "medusa-like" appearance. The presence of capsules on an organism gives its colonies a slimy consistency, and their absence a dry, roughened appearance. The colonies of some bacteria show coloring due to the elaboration of pigments by the organisms, but such pigments are not apparent in the individual cell under the microscope. Yellow and golden pigments are not uncommon, and others, including red, blue-green and violet, are met with from time to time.

Much stress should not be laid in all cases upon the morphology of masses of bacteria as an aid in distinguishing different species. The mass morphology, like the individual morphology, is subject to wide variation under varying conditions of life, and can be regarded as only one item in the sum total of characters that go to make up the concept of a bacterial species. One difference between colony types which has recently been discovered appears to be of deep-seated significance. In a number of bacterial groups it has been found that rough (R) and smooth (S) colonies will develop from a single, apparently pure, culture (p. 165). Under ordinary conditions, transfers from S and R colonies often breed true. The rough and smooth strains differ in virulence and other characteristics, and the marked and persistent association of colony characters with physiological and biochemical qualities may be of considerable biological significance.

BACTERIAL GROWTH

Although bacterial growth may be, and often is, assumed to be a simple process, both experimental evidence and theoretical considerations indicate that the problems of growth are relatively complex. It is convenient to consider bacterial growth under two general heads: first, the process of cell division or multiplication; and, second, the growth of bacterial populations.

Growth and Cell Division. By all odds the most common method of division is that of simple fission which divides the cell into approximately equal halves. Among bacilli and spirilla, cell division usually takes place at right angles to the long axis of the cell. The cocci may divide in one, two or three planes, thus giving rise to characteristic groupings such as chains, flat sheets or irregular masses, or cubical packets. Bacilli and spirilla show some elongation before division; cocci, as a rule, do not, although some exhibit an increase in the diameter of the cell without any alteration of its spherical form. The size which a single cell must reach before fission is, as among the higher forms of life, singularly constant for each species although, as will appear, some differences in this maximum size appear to be associated with the age of the culture.

Jour. Bact., 1949.) The center section is a light photograph of a young culture of *Bact. coli* fixed with osmium tetraoxide vapor but unstained. The chromatinic bodies are the light areas. (Robinow.) The lower section is also a light photograph of a 2½ hour culture of *Bact. coli*, fixed with osmium tetraoxide vapor and alcoholic mercuric chloride, hydrolyzed at 56°–60° C. with normal hydrochloric acid, and stained with Giemsa. Photographed with critical illumination after Köhler. The chromatinic bodies are clearly apparent as the dark areas. (Robinow.)

While bacterial multiplication is commonly observed to take place by simple transverse fission, other modes of cell division may occur. Some forms, such as the diphtheria bacillus and the tubercle bacillus, show Y-like splitting and true branching characteristic of some of the higher fungi. Budding, similar to the budding of yeast cells, has been reported by some observers. More complex processes, such as the formation within a mother cell of a number of viable granules, called gonidia, which, upon liberation, develop into typical bacillary forms, have been reported by other workers. The status of the great majority of these observations has been open to some question since many of the structures reported were too small to have been resolved by the light microscope. The electron microscope, however, allows the resolution of

Fig. 15. Electron micrograph of *Lactobacillus acidophilus* showing various stages in cell division. The initial slight constriction, deeper constriction, and partially separated daughter cells are all shown. Note the light cell walls surrounding the dense black protoplast, and the diploid cells connected by a delicate strand of protoplasm. (Mudd, Polevitsky and Anderson, SAB No. 78.)

very minute structures and with it Smith, Mudd and Hillier[22] have observed a kind of reproductive process in the Bacteroides (p. 540) and pleuropneumonia-like organisms (p. 548) in which these structurally fragile cells swell to form large round bodies that then may undergo multipolar germination. The outgrowing processes segment to form numbers of daughter cells. Whether such processes are of common occurrence among other kinds of bacteria is not clear.

The possibility of the occurrence of conjugation, and inferentially a sexual stage in reproduction, has been raised repeatedly but without adequate supporting evidence. Recently, however, Tatum and Lederberg[23] have reported the occurrence of mixed biochemical types of *Bact. coli* produced in mixed culture of strains derived from a single parent by x-ray irradiation and differ-

[22] Smith, Mudd and Hillier: Jour. Bact., 1948, 56:603.
[23] Tatum and Lederberg: Jour. Bact., 1947, 53:673; Lederberg: Genetics, 1947, 32:505.

ing in growth requirements. It is very difficult to interpret this evidence on other than the basis of a gene recombination arising through conjugation between the cell types. These observations have been confirmed by Haas, Wyss and Stone,[24] who have also observed an apparent fusion of chromatinic bodies on recovery from irradiation. Whether this last is to be interpreted as cytological evidence of a nuclear fusion and recombination of mutant characters is not clear.

While, then, a number of kinds of reproduction occur among the bacteria, binary fission is by far the most common. Under favorable conditions cell division may take place quite rapidly, as short a time as twenty minutes elapsing between one division and another of the colon bacillus. This rapidity of cell division is sometimes referred to as if it were a peculiar quality of bacteria, but as a matter of fact the embryonic cells of many higher forms of life divide quite as rapidly as bacteria. The remarkable thing about bacterial cell division is not so much the rapidity with which one cell division succeeds another as the fact that a very short time suffices for the growth of the young cell to maturity. A young bacterial cell attains full size and acquires the capacity to produce, in its turn, an independent organism much sooner than most other forms of life. This rapid reproduction of distinct individuals is plainly different from the multiplication of embryonic cells among higher organisms.

The Growth of Bacterial Populations. For obvious practical reasons bacteria are ordinarily manipulated and studied in groups consisting of large numbers of individuals. Likewise, bacterial growth is of practical significance, not in terms of one, two or three cell divisions, but .as growth in populations which often reach a density of three to four hundred million cells per cubic centimeter of culture fluid. Growth of these organisms in culture may be considered from a variety of points of view, the particular one adopted being dependent upon the nature of a given investigation. A simple view that is commonly, if tacitly, taken assumes that growth may be described by the reaction

$$\text{culture medium} + O_2 \longrightarrow \text{bacterial protoplasm} + \text{oxidized substances,}$$

which proceeds from left to right and eventually comes to an equilibrium. The reaction velocity constants may be determined either by the rate of disappearance of the reacting substances or the rate of appearance of end products. Although complicated by a variety of side reactions, the assumption of such a relation has been extremely useful in the studies on bacterial metabolism. The growth of autotrophic bacteria, for example, is conveniently measured in terms of the conversion of carbonate to the organic carbon of protoplasm. A somewhat more mechanistic concept is that of the instability of an inoculated culture medium which, after undergoing a series of complex changes, reaches a stable equilibrium at a considerably lower energy level. Such a concept is, of course, the basis of studies of the energy metabolism of bacteria. These and similar views are oversimplified and, although of undoubted value, present only limited aspects of the phenomena of bacterial growth.

[24] Proc. Nat. Acad. Sci., 1948, 34:229.

The existence of bacteria in large aggregations or *populations* is of no little significance because *ipso facto* they are subject to the principles of population mechanics. The potential multiplication of these organisms by geometrical progression may be realized only up to a certain point. As numbers increase in the microcosm of the test tube, competition between individual organisms for foodstuffs, oxygen and the like progressively reduces the opportunity for further growth until a saturating population is reached.

If no increasingly effective retardation were operative, the potential increase in a population would be expressed by the relation

$$\frac{dY}{dt} = bY$$

where Y is equal to the number of individuals per unit volume and b to the potential rate of multiplication of each organism. When, however, there is a maximal possible population, K, this potential geometric increase is only partially realized, the extent of the realization depending on how near the size of the population is to its maximum at any given moment. Or, mathematically,[25]

$$\frac{dY}{dt} = bY \frac{K - Y}{K}$$

This equation is the differential equation of the logistic curve the mathematical form of which is:

$$Y = \frac{K}{1 + e^{a - bx}}$$

This function, plotting as a symmetrical sigmoid curve, has been found to describe, with a high degree of precision, the growth of populations of a variety of organisms including man, yeasts, Drosophila, certain protozoa, etc.

If the numbers of bacteria in a growing culture are determined from time to time and plotted against time, the points fall on a similar sigmoid curve. The resulting curve is, however, asymmetrical in that the point of inflection is not halfway between the upper and lower asymptotes but is considerably near the lower asymptote. This discrepancy between bacterial populations and populations of other organisms has not as yet been satisfactorily explained.

Partly as a consequence of this discrepancy and partly as a result of the large error inherent in the enumeration of bacteria, the treatment of bacterial growth curves has taken a slightly different path. Such curves are ordinarily not plotted arithmetically but on semilog paper; *i.e.*, the logarithms of the numbers of bacteria are plotted against time. This procedure has the advantage that not only are the errors of enumeration minimized but, when the organisms are multiplying at a geometric rate, the points fall on a straight line and the generation time determines the slope of this line. Furthermore, the numbers of viable bacteria, as determined by plate count, decline not long after the maximum population has been reached and the organisms appear to die off at a geometric rate. The equilibrium predicted by the logistic function is

[25] For a more detailed discussion of such rationalization the student is referred to Lotka: *Principles of Physical Biology.* Williams & Wilkins Company, Baltimore. 1925.

not realized unless experimental conditions are such that the food supply is constantly renewed and the waste products are removed. Such an equilibrium is attained, for example, in cultures in constantly flowing culture medium and the viable count tends to attain and persist at a constant level though the total count continues to rise, *i.e.*, multiplication balances the death rate, but the dead cells accumulate.[26] A graphic representation of the rise and decline in numbers of viable bacteria in culture is given in Fig. 16.
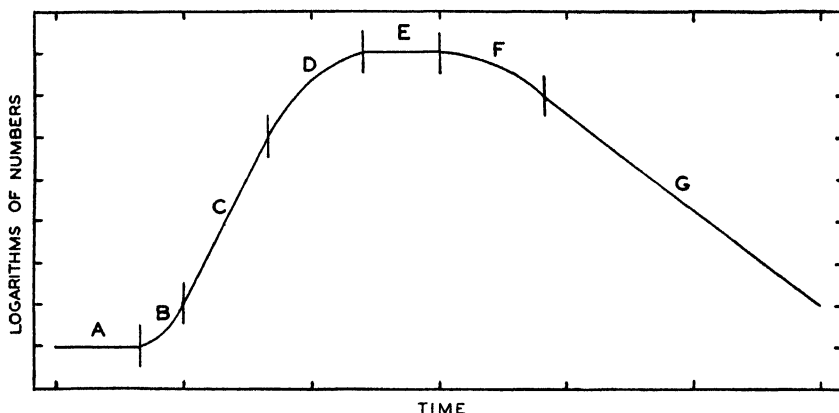
Fig. 16. Diagrammatic representation of a bacterial growth curve; numbers of viable organisms plotted against time of incubation. *A*, initial stationary phase; *B*, positive growth acceleration phase; *C*, logarithmic growth phase; *D*, negative growth acceleration phase; *E*, maximum stationary phase; *F*, phase of accelerated death; *G*, logarithmic death phase. (After Buchanan.)

Buchanan[27] has divided this curve up into seven parts which he designates as follows:

   A. *Initial Stationary Phase.* During this phase the number of bacteria remains constant.
   B. *Lag Phase or Positive Growth Acceleration Phase.* During this phase the generation time decreases progressively until a minimum is reached.
   C. *Logarithmic Growth Phase.* In this phase the generation time remains constant and the organisms increase in numbers by geometrical progression.
   D. *Negative Growth Acceleration Phase.* The generation time progressively increases during this phase and the bacteria continue to multiply but at a decreasing rate.
   E. *Maximum Stationary Phase.* Here the numbers of viable bacteria are at a maximum, neither increasing nor decreasing.
   F. *Phase of Accelerated Death.* Following the stationary phase, the numbers of viable bacteria decrease, slowly at first but with increasing rapidity, until a relatively constant rate it attained.
   G. *Logarithmic Death Phase.* During this phase a constant rate of death is maintained. It should be noted that this rate is not maintained indefinitely for the curve tends to become asymptotic to the X axis.

Different explanations have been offered to account for the varying growth rates observed in bacterial cultures. The failure of inoculated organisms to

[26] Jordan and Jacobs: Jour. Bact., 1944, 48:579.
[27] Buchanan: Jour. Inf. Dis., 1918, 23:109. See the review by Hinshelwood: Biol. Rev., 1944, *19*:150.

begin multiplication at the maximum rate at once has been attributed both to environmental factors and to the physiological state of the organisms. For example, it has been assumed that a partial pressure of carbon dioxide is essential to cell division, for, when it is prevented from accumulating by gassing with CO_2-free air or nitrogen, growth does not take place. On the other hand, the stationary and lag phases are said to be entirely eliminated by inoculation from a culture which is in the logarithmic phase of growth. Hinshelwood[28] has presented convincing evidence of a true and apparent lag phase, the latter resulting from the admixture in the inoculum of dead and dying cells with those capable of active proliferation. He suggests that the lag may be attributed to the time necessary for the accumulation of enzymes, diffusible coenzymes and essential intermediate compounds in the synthesis of cell substance (p. 185) to concentrations at which synthesis may occur at a maximum rate. The lag phase is of particular interest in connection with the action of drugs on bacteria (p. 152), since the bacteriostatic drugs produce an effect which is seemingly that of an indefinitely prolonged lag phase.

Hershey[29] has developed mathematical equations by which the latent period in multiplication and the numbers of organisms expected at any time during the phase of rapid growth may be predicted. Since only differences in the size of the cells are postulated (see below), the satisfactory agreement of experimental data with calculated values has undoubtedly more than accidental significance.

During the logarithmic growth phase the number of cells increases at a constant and practically exponential rate, for the mortality, under favorable conditions, is very low, more than 90 per cent of the cells continuing to divide. The rate of multiplication is independent of food concentration and of the effects of toxic substances over a relatively wide range, though at critical concentrations the activity of the latter increases sharply and contributes in large part to deceleration at the end of the logarithmic phase. The logarithmic growth phase, then, may be regarded as a steady state in which enzyme and substrate concentrations are maintained at such a level that the rate of synthesis of cell substance proceeds at the maximum for the strain of bacteria in that environment.

Likewise, the decreasing growth rate in the negative growth acceleration phase has been accounted for in various ways, including the rate of diffusion of oxygen into the medium, the accumulation of toxic by-products such as organic acids and ethyl alcohol, etc. It is probable that different factors are operative with different microorganisms and with the same microorganism under different conditions, and that each culture constitutes a special case.[30]

The mechanisms operative in bacterial death, both in culture and in the presence of antiseptics, are uncertain. The survivor curves are logarithmic, *i.e.*, the same percentage of viable organisms is dying at any particular moment. The fact that this rate may be described by a mathematical expression which likewise describes a monomolecular chemical reaction is generally held to be

[28] Hinshelwood: *Chemical Kinetics of the Bacterial Cell*. Clarendon Press, Oxford. 1946.

[29] Hershey: Jour. Gen. Physiol., 1939, 23:11.

[30] Henrici: *Morphologic Variation and the Rate of Growth of Bacteria*. Charles C Thomas, Springfield and Baltimore. 1928.

nothing more than a fortuitous coincidence, and the conclusion drawn by some workers that bacterial death is a monomolecular reaction has no basis in fact.

Morphologic Variation During Growth.[30] It has been observed that both the morphology and the physiological activity of bacteria differ in different phases of the growth of a culture. As growth begins to get under way, the maximum size that a given cell reaches before fission increases somewhat, and microscopic examination shows that a large proportion of the bacteria in a culture at this stage of development are appreciably larger than at other times. The cells stain evenly, and there is no evidence of granular structure even in those organisms in which metachromatic granules are most readily demonstrated. The rate of respiration per cell increases, reaching a maximum at the end of the phase of accelerating growth and declining as the culture goes into the logarithmic growth phase. The increased metabolic rate in the early growth stages is, however, more apparent than real and has been shown[31] to be quantitatively related to cell size. The cell size declines also as the culture goes into the logarithmic growth phase, although the individual cells remain homogeneous and take stains evenly. By the time the stationary phase is reached, the bacterial cells are uniformly smaller and in cultures of spore-forming organisms, many are forming spores. As the viable bacterial count decreases the cells no longer stain uniformly but show a granular structure and involutionary forms appear. This succession of morphologic types, the embryonic or young cells, the mature forms and finally the senescent forms, has been designated *cytomorphosis*.[30] It is possibly analogous to a similar succession of events in the higher forms of life.

THE CHEMICAL COMPOSITION OF BACTERIA

The chemical composition of bacteria is not greatly different from that of other living material. They differ in composition from species to species, and the chemical constitution of a given species is influenced to a considerable extent by the composition of the medium upon which the organisms are grown. In consequence, a precise statement of the relative amounts of the compounds and elements of which these organisms are composed is not possible; only crude approximations may be made.

Bacterial cells contain considerable quantities of water but somewhat less than the cells of the higher plants and animals. Estimates of water content vary widely, very likely owing to the difficulty of getting accurate wet weights. Although some organisms have been reported to contain as much as 90 per cent water, most bacteria show, in the hands of careful workers, 70 to 80 per cent water. In some species of bacteria an appreciable part, 17 to 28 per cent, of this is bound water.[32] The ash content, *i.e.*, the inorganic material remaining after combustion of the cells, depends to some degree, both qualitatively and quantitatively, on the inorganic content of the medium on which the organisms are grown. The values are, therefore, subject to considerable variation, and have been given as from 2 to 14 per cent of the dry weight of the cells. The ash is largely phosphoric acid, the P_2O_5 content ranging from 10 to 45 per cent of the total ash. Undoubtedly a considerable proportion of this

[31] Hershey and Bronfenbrenner: Jour. Gen. Physiol., 1938, *21:*721.
[32] *Cf.* Friedman and Henry: Jour. Bact., 1938, 36:99.

phosphorus exists in the cell in the form of nucleic acids. Sulfur, potassium, chlorine and calcium are also present in notable amounts, together with usually smaller quantities of magnesium, iron, silicon, etc.

There is a good deal better agreement regarding the carbon content of bacteria, for most analyses give about 50 per cent of the dry weight as carbon. A part of this, of course, is present in the cell as protein, but the polysaccharide gums which make up the capsular material account for a considerable portion. Cellulose is not a common constituent of bacteria but has been identified.[33] Hemicellulose is often present, and starchlike material (granulose) is found within the cells of some species.

The total nitrogen of bacterial cells varies more widely. An estimate of 8 to 15 per cent of the dry weight is in accord with the majority of analyses. A part of the nitrogen is present in the cell as protein, but very little is known of bacterial proteins. Both albumins and globulins have been isolated, the latter much more frequently. A fraction obtained by extraction by dilute alkali and called nucleoprotein has been of immunological interest and has been studied more thoroughly than other protein constituents. It appears to be a globulin combined with nucleic acid. In amino acid content the bacterial proteins do not differ materially from plant and animal proteins, and, in contrast with inorganic salt content, the amino acid composition of a given bacterium is a highly stable character and unaffected by variation in the culture medium.[34]

A finding in keeping with the high phosphorus content of bacterial ash is the relatively large proportion of the total nitrogen that may be accounted for as nucleic acid. These organisms contain a higher proportion of nucleic acid than any other tissue except thymus, a characteristic they share with the yeasts and molds. As much as 7.1 to 11.5 per cent of the total nitrogen is purine nitrogen. Adenine, guanine, cytosine and thymine have been isolated from the tubercle bacillus, the presence of thymine and the absence of uracil suggesting the animal type of nucleic acid. Guanine has been isolated from the colon bacillus and cytosine and uracil from the cholera vibrio. Thymine is absent from the latter, suggesting in turn the plant type of nucleic acid. In addition to the nucleic acids proper (polynucleotides), their decomposition products occur in the cell, including mononucleotides, nucleosides and the free bases. The structure of bacterial nucleotides is uncertain although the predominating base is adenine. The function of adenyl pyrophosphate as a phosphate carrier in fermentation is discussed elsewhere (p. 90).

Some bacteria contain considerable quantities of fats, lipids and waxes, *i.e.*, ether extractable material. The variation among bacteria as a group is great, analyses indicating that from 2 to 40 per cent of the dry weight may be lipoid in nature. Here, however, the variation within a species is not so great, but certain species, such as the acid-fast bacteria, contain large amounts of ether extractable material. These, for the most part waxes and complex alcohols, appear to be associated with the acid-fast staining properties of these organisms.

[33] Hibbert and Barsha: Jour. Amer. Chem. Soc., 1931, 53:3907.
[34] Stokes and Gunness: Jour. Bact., 1946, 52:195; Freeland and Gale: Biochem. Jour., 1947, 41:135.

Chapter 4

BACTERIAL PHYSIOLOGY[1,2]

The biochemical changes brought about through the activities of bacteria are by far the most obvious manifestations of their existence. The long-known but little understood phenomena of fermentation, putrefaction and decay, soil fertility and infectious diseases of man and lower animals are, in essence, no more than the exhibition of one or more of the many facets of the biochemical potentiality of these microorganisms. The oxidation of ammonia by the nitrifying bacteria, the decomposition of carbohydrates to alcohols and acids and the production of substances toxic to higher animals are a part of the normal activities of this heterogeneous group of living organisms. The elucidation of many of these phenomena has not only made possible a partial control of a not inconsiderable portion of man's environment but has provided an insight into the life processes of these organisms, organisms structurally so simple that morphology cannot carry us far in their study.

The term bacterial physiology, or, sometimes, bacterial metabolism, is a broad one which is generally assumed to include the entire sequence of events taking place in a bacterial culture. Since the small size of the individual bacterial cell precludes the utilization of many of the physiological techniques adapted to the multicellular, differentiated organism, the physiology of bacteria becomes, for all practical purposes, a biochemical physiology. The very magnitude of the biochemical changes brought about by these organisms tends to reinforce a belief in their relative importance; bacteria have, for example, been shown to consume forty to sixty times as much energy as man in terms of calories per gram of body nitrogen. The obvious significance of physiological activities is so readily apparent that it has tended to obscure to many the undoubted importance of the environmental factors that influence the functioning of the living cell. It is to be borne in mind, therefore, that although bacterial physiology is a branch of general cellular physiology, it must be regarded as a special case which, in many instances, goes considerably further afield into other fundamental aspects of biology than the present concept of cellular physiology is generally assumed to do.

---

[1] For general and more detailed discussions see: Buchanan and Fulmer: *Physiology and Biochemistry of Bacteria,* Vols. I, II and III. Williams and Wilkins Company, Baltimore. 1928; Stephenson: *Bacterial Metabolism,* 2nd ed., Longmans, Green, London. 1939; Porter: *Bacterial Chemistry and Physiology,* John Wiley and Sons, New York. 1946; and the successive volumes of Annual Review of Biochemistry, Annual Review of Microbiology, Ergebnisse der Enzymforschung and Advances in Enzymology.

[2] The sections on respiration and carbohydrate metabolism were written by Dr. J. W. Moulder, who also revised the section on autotrophic metabolism.

Morphologically bacteria are simple organisms and are often regarded as a primitive type of cell. These organisms are not simple physiologically, however; many of them oxidize glucose, for example, by mechanisms very similar to and often more complex than those used by higher animals. Others, such as the nitrifying bacteria, whose respiratory processes consist of but a single simple oxidation, synthesize all the components of protoplasm from inorganic compounds of carbon and nitrogen—a more complex process than that of joining together preformed amino acids. Since these organisms metabolize in these and other highly complex ways, it is difficult to regard them as physiologically simple. There is, however, some reason to think that bacteria are primitive cells. The view that the pathogenic organisms are degenerate forms cannot be taken to imply that the free-living forms are likewise degenerate with respect to other living cells, for such a postulate entails the assumption that the physiological capabilities of these organisms are acquired characters, and is not in keeping with current knowledge of biology. The multiplicity of reactions which bacteria are able to bring about suggests that, in these organisms, the original capabilities of protoplasm have not been lost to the extent that they have been by the differentiated cells of higher organisms. The animal body, for example, has no mechanism for the breakdown of cellulose, yet bacteria having the ability to hydrolyze this substance are universally distributed in the soil. Bacteria capable of breaking down a particular organic compound have been isolated since the early days of bacteriology by the relatively simple expedient of inoculating a highly specific medium containing the substance under consideration as the only source of energy with a complex mixture of organisms such as exist in samples of soil. It is probable that by this method a bacterium could be found which would oxidize almost any organic compound. No group of higher organisms shows such a high degree of physiological flexibility. The wide range of carbon compounds that a single bacterial species may oxidize and the multiplicity of end products of the fermentation of a single carbohydrate likewise point toward such a flexibility. The simplicity and variability of the nutritive requirements of many bacteria also suggest a physiologically primitive cell.

The physiological economy of the cell is dynamic rather than static, and life is compatible only with a state of greater or lesser, but nevertheless continuous, physiological activity. This activity may be approximately separated into two phases, the exothermic, oxidative, energy-yielding reactions, and the endothermic reduction reactions of synthesis. The former make possible a continuous, regulated supply of energy to the cell, while the latter channel this energy, or a part of it, into the synthesis of cell substance. The living cell may, therefore, be regarded as a machine for the manufacture of protoplasm, operating with a greater or lesser degree of efficiency, whose energy is supplied by the complex process of respiration. This oxidation-reduction process is not complete within itself, *i.e.*, the machine is not 100 per cent efficient, and the respiratory oxidations are not precisely balanced by the reductions of synthesis. Other substances must, therefore, be reduced to balance the excess of oxidation reactions and this is carried out through a series of reactions leading to the eventual reduction of atmospheric oxygen. The separation of the processes of respiration and synthesis cannot be complete, of course, for in many instances

the products of the respiratory process are assimilated by the cell and utilized for synthetic purposes; in fact in very many instances a preliminary decomposition is necessary before assimilation can occur.

From the point of view of chemical kinetics it is tempting to regard the living cell as a macromolecular polyfunctional free radical system which is partially stable because of spatial separation of the free valencies in the organized cell structure that would otherwise saturate one another in a homogeneous phase. The structural protection is only partial, however, and decay of the system is counteracted by its participation in chain reactions accompanied by large decreases in free energy, *i.e.*, free valencies are maintained by the respiratory reactions.

A reasonable amount of information is available with respect to respiration, regarding both the catalysis of the reactions and the intermediary metabolism of the substrates, *i.e.*, the successive reactions involved. On the other hand, the mechanisms of synthesis of the components of protoplasm are almost completely unknown, not only for the bacteria but for other organisms as well. Elucidation of the nutritive requirements of bacteria may, perhaps, be regarded as definition of the precursors of protoplasm in the catalytic system that is the microorganism under consideration; it may equally well be regarded as definition of the limits of the synthetic abilities of the organism.

RESPIRATION[3]

Lavoisier's classic experiments on animal respiration performed at the close of the eighteenth century led to the definition of respiration as the utilization of oxygen and the production of carbon dioxide and water, and the presence of molecular oxygen was considered to be absolutely essential for life. However, the meaning of the term respiration was gradually broadened after Pasteur discovered in 1861 that many bacteria can grow and thrive in the complete absence of oxygen. Perhaps the most general definition one can give is that of respiration as the sum total of the chemical reactions carried out by the living cell which result in the liberation of energy.

Of these energy-yielding reactions by far the most important are those of oxidation-reduction. In an oxidation-reduction reaction, one substance is oxidized and one substance is reduced. According to Clark[4] and to Michaelis,[5] the oxidized substance loses electrons and the reduced substance gains electrons. If, in the respiration of a living cell, molecular oxygen is the ultimate oxidizing agent, and thus the ultimate acceptor of electrons, the respiration is said to be aerobic. If another substance, not oxygen, is the final electron acceptor, the respiration is said to be anaerobic. The oxidation-reduction reactions of respiration have two functions: they transform nutrient material into substances needed for the maintenance and growth of the cell, and they liberate energy from the nutrient material for use in the cellular economy.

[3] For a general discussion of respiration, see Green: *Mechanisms of Biological Oxidation*. Cambridge University Press, Cambridge. 1940; and *A Symposium on Respiratory Enzymes*. University of Wisconsin Press, Madison. 1942. The general subject of bacterial respiration has been discussed by Werkman: Bact. Rev., 1939, 3:187.

[4] Clark: Public Health Repts., 1923, 38:443. This and nine additional papers on oxidation-reduction studies are contained in Hygienic Laboratory Bulletin No. 151, 1928.

[5] Michaelis and Schubert: Chem. Rev., 1938, 22:437.

Oxidation-Reduction Potential.[6] A simple example of an oxidation-reduction reaction is the reaction between ferric iron and cuprous copper.

$$Fe^{+++} + Cu^+ \rightarrow Fe^{++} + Cu^{++}$$

$$:\overset{..}{Fe} + :\overset{..}{Cu}\cdot \rightarrow :\overset{..}{Fe} + :\overset{..}{Cu}$$
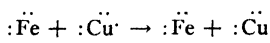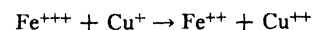
When the oxidation-reduction involves organic compounds, as in almost all biological oxidations, oxidation often results in transfer of hydrogen atoms as well as of electrons, and biological oxidation is often referred to as hydrogen transport. The oxidation of ethanol to acetaldehyde, for example, is accompanied by transfer of hydrogen as well as of electrons.

$$\overset{\displaystyle H}{\underset{\displaystyle H}{H_3C : \overset{..}{C} : \overset{..}{O} : H}} \rightleftharpoons H_3C : \overset{..}{C} : : \overset{..}{O} : + 2\,e + 2\,H^+$$
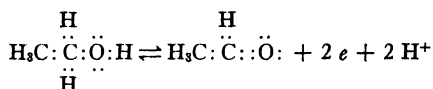
It is obvious that any substance capable of entering into oxidation-reduction reactions must exist in two forms—an oxidized form and a reduced form. These two forms constitute a reversible oxidation-reduction system.

$$Ox \rightleftharpoons Red + n\,e$$

Oxidized form   Reduced form

The oxidizing power of an oxidation-reduction system is a function of its ability to donate electrons to another system and may be quantitatively expressed as the oxidation-reduction potential of the system.

The oxidation-reduction potential has the dimensions of volts and may be measured in a suitable electrical circuit. The oxidation-reduction potential, E_h, is defined by the general electrode equation,

$$E_h = E_o - \frac{RT}{nF}\,\ln\frac{[Red]}{[Ox]}$$

in which E_h and E_o are measured in volts, R is the gas constant, T is the absolute temperature, F is the faraday, and n is the number of electrons transferred in the oxidation-reduction reaction. The observed oxidation-reduction potential, E_h, is a function of E_o, a constant for the system, and the ratio of the molar concentrations of the oxidized and reduced forms. When

$$\frac{[Red]}{[Ox]} = 1, \qquad then \qquad E_h = E_o$$

and this relationship serves to define the constant, E_o. Each oxidation-reduction system has a characteristic E_o value. The potential of the system,

$$H_2 \rightleftharpoons 2\,H^+ + 2\,e$$

at pH 0 and 1 atmosphere of hydrogen is arbitrarily assumed to be 0, and all other oxidation-reduction potentials are expressed in relation to the potential of this system, the normal hydrogen electrode.

If either or both the oxidized or reduced forms of a system are ionized, changes in pH will alter the potential of the system by disturbing ionic equilibria. Because of this effect, the pH must be specified for each observed value of E_h, or else the value is meaningless. The E_o of a system represents the potential at which the system is half-oxidized at pH 0, and another symbol, E_o', is used to represent the potential of a half-oxidized system at a specified pH.

[6] For a general discussion appropriate here, see Hewitt: *Oxidation-Reduction Potentials in Bacteriology and Biochemistry.* 4th ed. London County Council. P. S. King & Son, Ltd., London. 1936.

Systems with relatively high E_o's have strong tendencies to accept electrons and are good oxidizing agents. Systems with relatively low E_o's have strong tendencies to donate electrons and are good reducing agents. The oxidized form of any system may oxidize (accept electrons from) the reduced form of any system with sufficiently lower E_o. Such reactions always proceed with the liberation of energy (decrease in the free energy of the system). That is, the energy contained in the products of the reaction is always less than that contained in the reactants.

Application to Biological Systems. The foregoing treatment of oxidation-reduction potential applies only to completely reversible systems. A number of biological systems are reversible and electromotively active and therefore susceptible to such analysis. This group includes the cytochromes and cytochrome oxidase, the flavoproteins, and a number of naturally occurring pigments. A second group is made up of the sluggish systems, so called because of their sluggish behavior at the electrode, which are regarded as only partially electromotively active. The pyridine nucleotides and the sulfhydryl compounds such as glutathione and cysteine belong to this group, and the measurement of their oxidation-reduction potentials is difficult and generally unsatisfactory. Lastly, there are those sluggish systems that develop potentials only in the presence of specific enzymes, the enzymatic oxidation-reduction systems. There are many of these, including succinic acid-fumaric acid, lactic acid-pyruvic acid, alcohol-acetaldehyde, and others.

Since many of the oxidation-reduction systems in the living cell fall into this last group, it is very difficult to interpret potentials produced during active cellular metabolism when a number of systems must be functioning simultaneously. As a matter of fact, it is doubtful that the reducing intensities developed should be termed "oxidation-reduction potentials" at all. Probably reduction potential or reducing intensity is the most satisfactory term for potentials developed by the actively metabolizing intact cell. The accompanying table gives the oxidation-reduction potentials of some important biological systems.

OXIDATION-REDUCTION POTENTIALS OF BIOLOGICALLY IMPORTANT SYSTEMS AT pH 7

Oxidation-Reduction System		$É_o$ in volts	T C°
Oxidized Form	*Reduced Form*		
O_2	H_2O	0.81	25
Ferri-cytochrome oxidase	Ferro-cytochrome oxidase	?	
Ferri-cytochrome *a*	Ferro-cytochrome *a*	0.29	25
Ferri-cytochrome *c*	Ferro-cytochrome *c*	0.26	25
Ferri-cytochrome *b*	Ferro-cytochrome *b*	—0.04	25
NO_3^-	NO_2^-	0.05	30
Methylene blue	Reduced methylene blue	0.01	30
Pyocyanine	Reduced pyocyanine	—0.03	30
Fumaric acid	Succinic acid	—0.03	25
Flavoprotein	Reduced flavoprotein	—0.06	38
Oxalacetic acid	Malic acid	—0.10	37
Phthiocol	Reduced phthiocol	—0.17	30
Pyruvic acid	Lactic acid	—0.18	35
Acetaldehyde	Ethanol	—0.19	30
Diphosphopyridine nucleotide	Reduced diphosphopridine nucleotide	—0.29	30
H+	H_2	—0.41	25

Bacterial Physiology

Reduction Potentials of Bacteria. The development of reducing properties by bacteria is associated with equivalent electrometric and colorimetric changes, indicating that the predominant system or systems are reversible. The potentials may be developed by bacterial suspensions in the presence of added substrate, or during growth in culture; the latter state has been by far the more commonly studied.
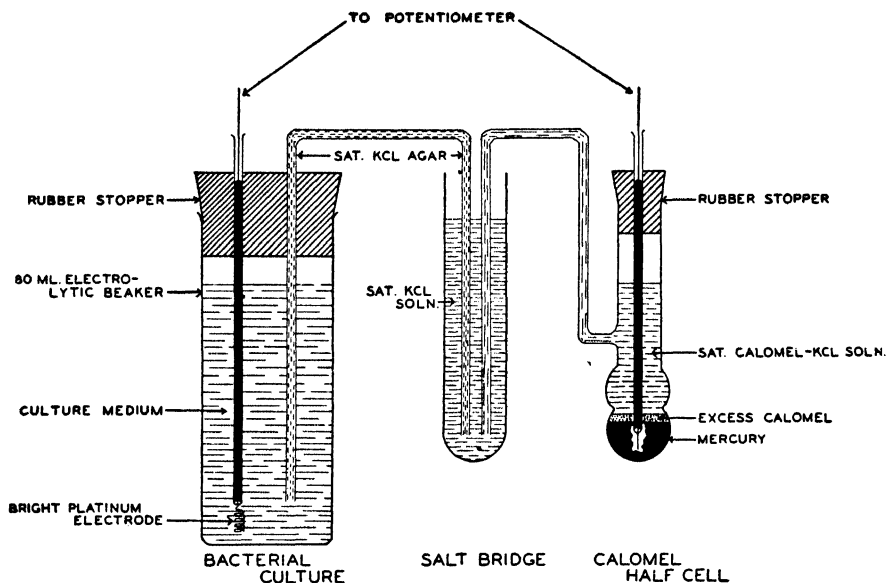
Fig. 17. Diagrammatic representation of the method used in the measurement of reduction potentials developed in bacterial cultures. The culture vessel should contain duplicate electrodes and an additional opening for inoculation, removal of samples, etc.

Measurements of the reducing intensity are made electrometrically, using bright platinum or gold-plated platinum electrodes, as the use of indicator dyes which change color with oxidation or reduction has been unsatisfactory. A satisfactory arrangement is illustrated in Fig. 17 in diagrammatic form. The bacterial culture is a half cell, and the standard half cell is a saturated calomel cell. The whole is immersed in a constant temperature bath. With buffered broth as the culture medium, the pH is maintained without significant variation. The reducing capacity of the bacterial culture is very low, *i.e.*, it is poorly poised (analogous to poorly buffered with respect to changes in pH), and if appreciable amounts of current are passed through the system in the measurement of the potential, the electrodes polarize, making readings impossible. It is necessary, therefore, to insert a high resistance in series with the cell, so that only a very small current passes through the culture. The calculation of the potential is simple:

$$E_h = E_{obs} + E_{std}$$

where E_{obs} is the observed potential and E_{std} is the potential of the standard half cell. Measurement of the developing potential may be made at intervals to give a time-potential curve.

The potential of a freshly-prepared sterile culture medium often shows a slight, gradual negative drift. When inoculated with bacteria, however, the

potential falls rapidly during the early hours of incubation. Thereafter, it differs somewhat with the type of bacterium. In the case of the obligate anaerobes (see Fig. 18) it reaches and maintains a very low level, sometimes approaching or reaching the level of hydrogen overvoltage, *i.e.*, more negative than the normal hydrogen electrode. Some facultative anaerobes such as the colon bacillus reach similar low levels while others do not. Other kinds of bacteria behave still differently. Pneumococcus cultures, for example, show a rapid positive drift after a negative potential has been established; this is probably due to the accumulation of peroxide since it does not occur in catalase broth cultures. Representative time-potential curves are given in Fig. 18.
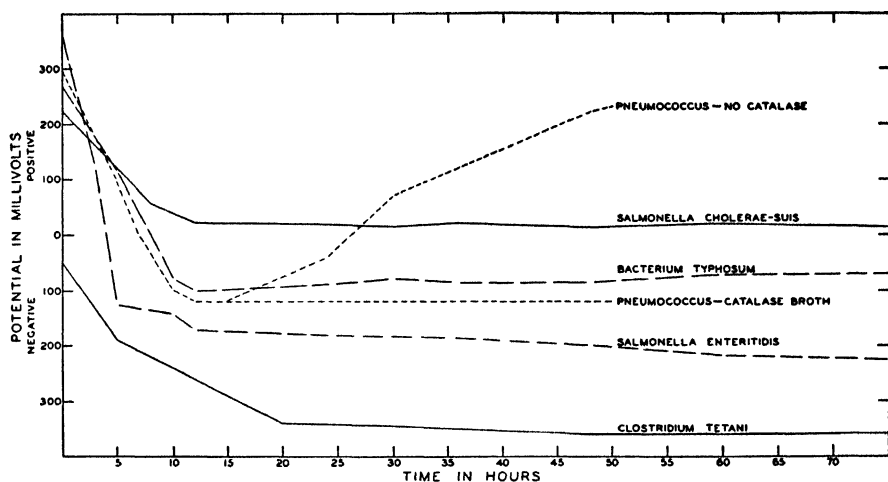
Fig. 18. Time-potential curves illustrating the development of reducing intensities in bacterial cultures. Note the rise in potential in pneumococcus cultures in non-catalase-containing media resulting from the accumulation of peroxide. The development of species-characteristic potentials is apparent. Pneumococcus data from Hewitt, *Cl. tetani* from Gillespie and Rettger, remainder from Burrows and Jordan.

Mechanism of Biological Oxidation.[7] The importance of hydrogen transfer in biological oxidations was early recognized and particularly emphasized in the work of Thunberg and Wieland. Wieland postulated that in biological oxidation-reductions the hydrogen atoms of substrates were activated by specific enzymes called dehydrogenases; once activated, these hydrogen atoms were then spontaneously transferred to any suitable hydrogen acceptor. However, at about the same time, Warburg showed that oxygen may be similarly activated by an iron-porphyrin enzyme (*Atmungsferment*) and postulated that activated oxygen could spontaneously accept hydrogen from substrate molecules. The ideas of Wieland and Warburg were soon harmoniously reconciled in the now generally accepted view of biological oxidation in which substrate hydrogen is activated by dehydrogenases and molecular oxygen

[7] In addition to the general references given in 3, see also Goddard's article "The Respiration of Cells and Tissues" in Hober: *Physical Chemistry of Cells and Tissues*. The Blakiston Company, Philadelphia. 1945; and Oppenheimer and Stern: *Biological Oxidation*. Nordemann Publishing Co., Inc., New York. 1939.

acts as a hydrogen acceptor only after activation by Warburg's respiratory enzyme.[8] The dehydrogenases of Wieland have been found to consist of a specific enzyme protein and a low molecular weight coenzyme which undergoes reversible oxidation-reduction. The respiratory enzyme of Warburg functions in respiration by catalyzing the oxidation of other iron-porphyrin protein enzymes, the cytochromes, by molecular oxygen, and for this reason has been given the functional name, cytochrome oxidase. Interposed in the train of hydrogen and electron transport between the dehydrogenases and the cytochromes is another type of respiratory enzyme, the flavoprotein, which is reduced by the dehydrogenases and oxidized by the cytochromes. Thus, there is a clear and continuous pathway for hydrogen and electron transport from the substrate to molecular oxygen, which is illustrated in the accompanying diagram.

<div align="center">AEROBIC OXIDATION OF SUBSTRATES</div>

Substrate
$\updownarrow$
Substrate-H_2 + Pyridino-
            protein
            $\updownarrow$
            Pyridino-
            protein-H_2 + Flavo-
                        protein
                        $\updownarrow$
                        Flavo-
                        protein-H_2 + 2 Cyto-
                                    chrome Fe^{+++}
                                    $\updownarrow$
                                    2 Cyto-
                                    chrome Fe^{++} + 2 Cytochrome
                                                oxidase Fe^{+++}
                                                $\updownarrow$
                                                2 Cytochrome
                                                oxidase Fe^{++} + ½ O_2
                                                            $\downarrow$
                                                            H_2O

<div align="center">*Net Reaction*</div>

Substrate-H_2 + ½ O_2 $\longrightarrow$ Substrate + H_2O
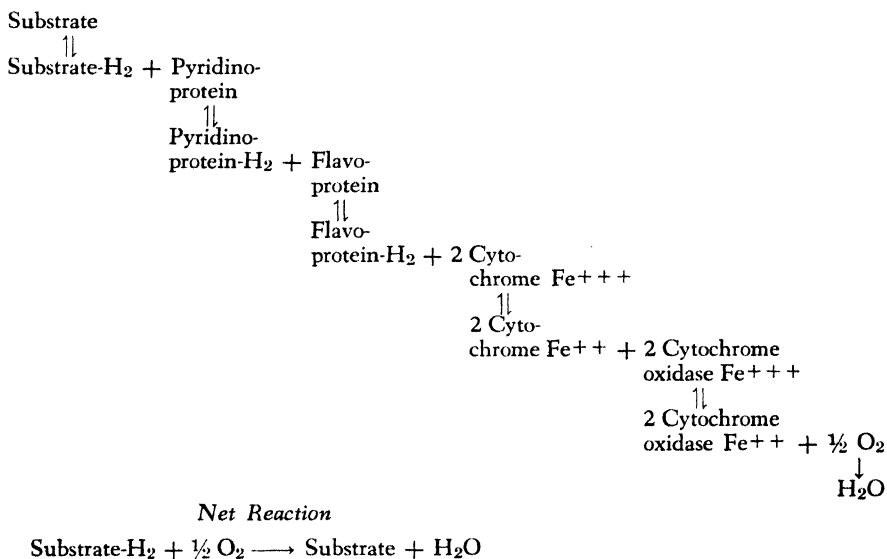
In most aerobic organisms this system is complete and functional, but in facultative and obligate anaerobes, portions of the hydrogen transport chain may be absent or non-functional (*vide infra*). In anaerobic respiration, the hydrogen and electrons of substrates are not transferred to molecular oxygen but to other substrates instead. The iron-porphyrin catalysts are not involved in anaerobic respiration, but the flavoproteins may sometimes act as intermediate carriers.

Respiratory Enzymes in Bacteria.[9] Although bacteria contain very active respiratory enzymes, much less is known about their chemical nature than about similar enzymes in yeast and in the tissues of higher animals,

[8] Kluyver and Donker: Chem. Zelle Gewebe, 1926, *13*:134.
[9] See Porter: *Bacterial Chemistry and Physiology*. John Wiley and Sons, Inc., New York. 1946. Chapter 6.

chiefly because it has been very difficult to obtain active enzyme extracts from bacterial cells.

Dehydrogenases. Dehydrogenases consist of a specific protein, or apo-enzyme, combined with a reversibly oxidized and reduced coenzyme. They oxidize metabolites and are in turn reoxidized by other enzymes, not by molecular oxygen. Dehydrogenases possess a high degree of specificity for the substrate which they oxidize, and are usually named on the basis of their substrate specificity, *i.e.*, lactic dehydrogenase, succinic dehydrogenase, etc. This substrate specificity is a function of the protein component, since a single coenzyme may be a part of several different dehydrogenases.

The specific protein combines reversibly with both coenzyme and substrate. The actual oxidation-reduction probably occurs while all three are in physical combination.

$$\text{coenzyme} + \text{apoenzyme} \rightleftharpoons \text{coenzyme} - \text{apoenzyme}$$
$$(\text{dehydrogenase})$$
$$\text{coenzyme} - \text{apoenzyme} + \text{substrate-H}_2 \rightleftharpoons$$
$$\text{coenzyme} - \text{apoenzyme} - \text{substrate-H}_2$$
$$\text{coenzyme} - \text{apoenzyme} - \text{substrate-H}_2 \rightleftharpoons$$
$$\text{coenzyme-H}_2 - \text{apoenzyme} - \text{substrate}$$
$$\text{coenzyme-H}_2 - \text{apoenzyme} - \text{substrate} \rightleftharpoons$$
$$\text{coenzyme-H}_2 - \text{apoenzyme} + \text{substrate}$$
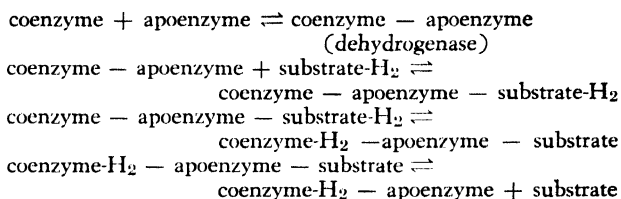
Two general types of dehydrogenases are found in bacteria and in other organisms. The *pyridinoproteins* are dehydrogenases containing either di- or triphosphopyridine nucleotide (DPN or TPN) as coenzymes. The provisional structural formula of DPN (coenzyme I, cozymase) is illustrated.
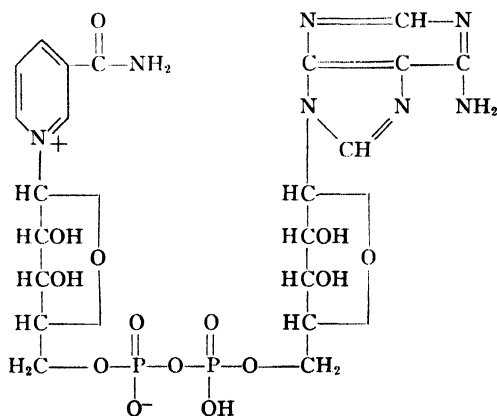
diphosphopyridine nucleotide

The structure of TPN (coenzyme II) is identical with that of DPN except for the presence of an additional molecule of phosphoric acid which is probably esterified to one of the hydroxyl groups of the pentose residue. Only the pyridine ring of both DPN and TPN undergoes reversible oxidation-reduction as indicated in the accompanying equation.

oxdized form   *reduced form*

$$+ 2H^+ + 2e \rightleftharpoons \quad\quad + H^+$$
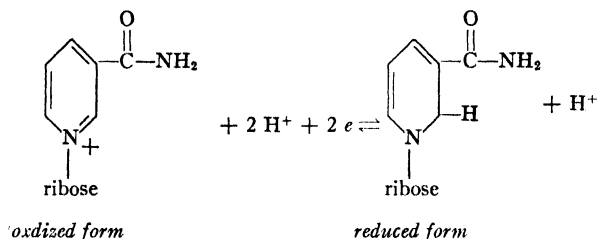
The pyridinoproteins are soluble, relatively easily extracted from cells, and their reduced forms are reoxidized by flavoproteins. In bacteria, the malic, lactic and 3-phosphoglyceraldehyde dehydrogenases are DPN-proteins, while glutamic acid dehydrogenase is a TPN-protein. The *cytochrome-linked* dehydrogenases are associated with the insoluble submicroscopic cell particles. In crude preparations they react with oxygen through the cytochrome system, but there is evidence that an additional hydrogen carrier is involved. It may be a flavoprotein or perhaps cytochrome *b*. The succinic and formic dehydrogenases of *Bact. coli* and the lactic dehydrogenase of the gonococcus are cytochrome-linked.

The oxidation of substrates by dehydrogenases may be conveniently studied in the absence of other respiratory enzymes by means of a technique introduced by Thunberg. In this procedure, the dehydrogenase is reduced by its specific substrate and reoxidized by a reversibly oxidized and reduced dye possessing a suitable oxidation-reduction potential.

$$\text{substrate-}H_2 + \text{coenzyme} - \text{apoenzyme} \rightleftharpoons$$
$$\text{coenzyme-}H_2 - \text{apoenzyme} + \text{substrate}$$
$$\text{coenzyme-}H_2 - \text{apoenzyme} + \text{dye} \rightleftharpoons$$
$$\text{coenzyme} - \text{apoenzyme} + \text{dye-}H_2$$
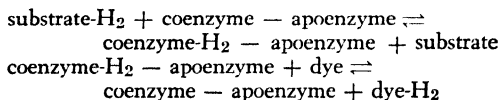
When the oxidized form of the dye is colored and the reduced form is colorless (methylene blue, for example), the rate of such a reaction may be measured in terms of the rate of decolorization of the dye in the absence of oxygen.

Flavoproteins. The flavoproteins are respiratory enzymes containing either riboflavin phosphate or flavin adenine dinucleotide as coenzymes.

riboflavin phosphate
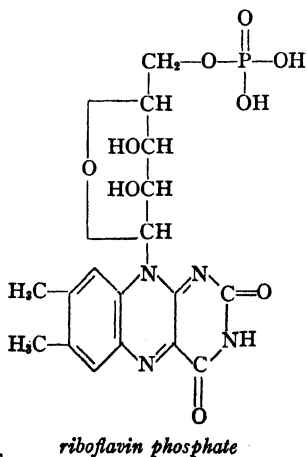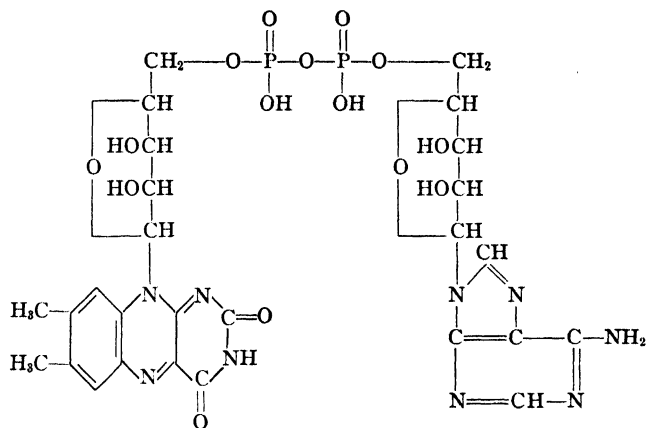
flavin adenine dinucleotide

In both riboflavin coenzymes, oxidation-reduction is confined to the isoalloxazine nucleus.
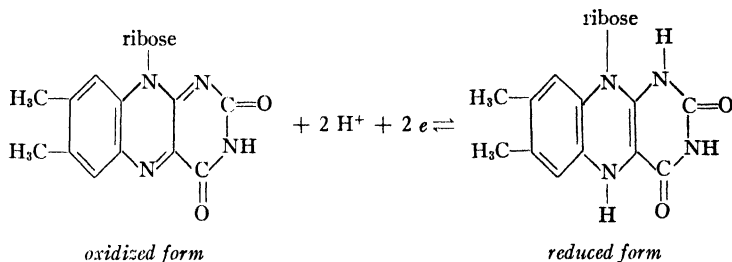
oxidized form   *reduced form*

Derivatives of riboflavin are apparently present in all bacteria and are found in especially large amounts in some anaerobes. Flavoprotein enzymes fall into two functional groups. The *oxidases* catalyze irreversible oxidations of substrates and react directly with oxygen to form hydrogen peroxide.

$$\text{substrate-}H_2 + \text{flavoprotein} \rightleftharpoons \text{substrate} + \text{flavoprotein-}H_2$$
$$\text{flavoprotein-}H_2 + O_2 \longrightarrow \text{flavoprotein} + H_2O_2$$
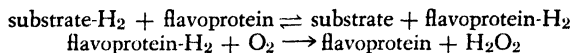
These flavoprotein oxidases have been studied almost entirely in animal tissues, and no clear-cut demonstration of such an enzyme in bacteria has been made. *Lactobacillus delbrueckii* contains an auto-oxidizable flavoprotein which is probably responsible for the oxygen uptake of this cytochrome-free organism, but the flavoprotein has not been shown to react directly with metabolites. Most peroxide formation in bacteria is probably due to flavoprotein-catalyzed reactions.

Other flavoproteins function in hydrogen transport as the link between the dehydrogenases and the cytochromes.

$$\text{pyridinoprotein-}H_2 + \text{flavoprotein} \rightleftharpoons$$
$$\text{pyridinoprotein} + \text{flavoprotein-}H_2$$
$$\text{flavoprotein-}H_2 + 2 \text{ cytochrome Fe}^{+++} \rightleftharpoons$$
$$\text{flavoprotein} + 2 \text{ cytochrome Fe}^{++} + 2 H^+$$
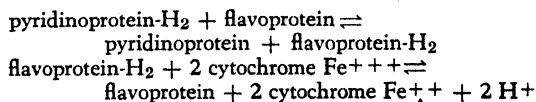
These flavoproteins catalyze reversible reactions and do not react with either metabolites or molecular oxygen. Flavoproteins of this type have been studied in *Bact. coli,* and they probably are found in all cytochrome-containing bacteria.

Iron-Porphyrin Protein Enzymes. Several important respiratory enzymes have iron-porphyrin compounds as coenzymes. These iron-porphyrins are closely related to, but not identical with, the heme of hemoglobin. Only the iron atom of the iron-porphyrin undergoes reversible oxidation-reduction. Almost all aerobic cells contain three iron-porphyrin protein pigments, the *cytochromes a, b* and *c.* Each of the cytochromes may readily be identified spectroscopically by four sharp absorption bands in the visible spectrum when in the reduced ferrous state, and their presence or absence in a relatively large number of bacteria has been determined.

The cytochromes are almost invariably present in aerobic bacteria and absent in obligate anaerobes, while facultative anaerobes may be lacking one, two or all of the cytochromes. Although some cytochrome-free bacteria, such as *L. delbrueckii,* rapidly consume oxygen, there is no doubt that the cytochromes are intimately associated with sustained aerobic growth and metabolism.

Cytochrome oxidase, Warburg's respiratory enzyme, is a portion of the sub-microscopic particulate structure of the cell, but it may be brought into solution or stable suspension by treatment with ultrasonic vibrations. Although cytochrome oxidase has never been isolated in pure form, its absorption spectrum and its behavior with inhibitors show that it is an iron-porphyrin protein.

The cytochrome system functions in respiration as the last link between substrate and oxygen. The path of the electron and hydrogen transport over the cytochrome system may be divided into three steps:

1. Reduction of cytochrome
    flavoprotein-H_2 + 2 cytochrome $Fe^{+++} \rightleftharpoons$
        flavoprotein + 2 cytochrome Fe^{++} + 2 H^+
2. Reduction of cytochrome oxidase
    cytochrome Fe^{++} + cytochrome oxidase $Fe^{+++} \rightleftharpoons$
        cytochrome Fe^{+++} + cytochrome oxidase Fe^{++} ᛁ
3. Reduction of oxygen
    cytochrome oxidase Fe^{++} + ½ O_2 + 2 $H^+ \rightarrow$
        cytochrome oxidase Fe^{+++} + H_2O

No peroxide can be detected in oxidations catalyzed by the cytochrome system. The mechanism of the reaction between cytochrome oxidase and oxygen (activation of oxygen) is unknown, but Warburg has suggested that oxygen forms a loose combination with cytochrome oxidase similar to oxyhemoglobin; in this complex, the oxygen is activated and accepts electrons and hydrogen to form water. The oxygen consumption of almost all cells is more or less completely inhibited by hydrogen cyanide and carbon monoxide, and the carbon monoxide inhibition is removed by irradiation with visible light. Only the iron-porphyrins combine with both hydrogen cyanide and carbon monoxide, the carbon monoxide compounds being light-dissociable. The importance of the cytochrome system in aerobic respiration is shown by the almost complete inhibition of oxygen uptake by cyanide or carbon monoxide in cytochrome-

containing cells. It has been found that in respiring yeast cultures each molecule of cytochrome is oxidized and reduced 4000 times a minute, a rate sufficient to account for all the oxygen uptake of the cultures. The portion of bacterial respiration not inhibited by cyanide or carbon monoxide is probably carried out by auto-oxidizable flavoproteins or by other respiratory enzymes with reversibly oxidized and reduced substances such as phthiocol, a yellow pigment of the tubercle bacillus, or pyocyanin, a pigment produced by *Ps. pyocyanea*, as coenzymes.

Most organisms contain an enzyme called *catalase* which decomposes hydrogen peroxide.

$$2 H_2O_2 \longrightarrow 2H_2O + O_2$$
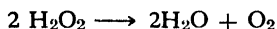
The catalase crystallized from beef liver by Keilin and Hartree is an iron-porphyrin protein and it is generally accepted that catalase from other sources has a similar chemical nature. Catalase is present in almost all aerobic bacteria and some facultative anaerobes, but under usual conditions of culture it cannot be detected in Clostridium and some of the streptococci, lactic acid bacteria and dysentery bacteria.

The *peroxidases* are enzymes, probably iron-porphyrin proteins, which catalyze the oxidation of substrates by hydrogen peroxide. Their distribution in bacteria parallels that of the cytochromes and catalase, and their function in bacterial metabolism is unknown.

Hydrogenase[10] is a bacterial enzyme which catalyzes the reaction of the normal hydrogen electrode.

$$H_2 \rightleftharpoons 2 H^+ + 2 e$$

In the presence of hydrogenase and molecular hydrogen, many substrates are reduced.

$$2 H_2 + O_2 \longrightarrow 2 H_2O$$
$$H_2 + HNO_3 \longrightarrow HNO_2 + H_2O$$
$$4 H_2 + H_2SO_4 \longrightarrow H_2S + 4 H_2O$$
$$4 H_2 + CO_2 \longrightarrow CH_4 + 2 H_2O$$
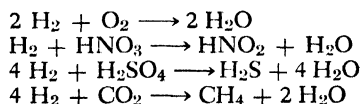
Hydrogenase activity was first found in the autotrophic hydrogen bacteria and much later in many heterotrophic facultative anaerobes such as *Bact. coli.* Recently, hydrogenase has been observed in the strongly aerobic Azotobacter where it is apparently involved in some manner with nitrogen fixation. Although hydrogenase has never been isolated, it is probably an iron-porphyrin protein because it is inhibited by hydrogen cyanide and by carbon monoxide, the latter inhibition being light-reversible, and because it is not found in cultures grown on iron-deficient mediums. Hydrogenase is inactivated by molecular oxygen, and it has been suggested that the Fe^{++} form is active while the Fe^{+++} form is inactive.

The Relation of Bacteria to Molecular Oxygen. The bacteria differ from one another in their relationship to molecular oxygen. Certain bacteria, the *obligate aerobes*, require ready access to air, growing feebly or not at all

[10] Recent investigations on hydrogenase are discussed in considerable detail by Lipmann: Ann. Rev. Biochem., 1943, *12*:5; and Stephenson: Antonie van Leeuwenhoek, 1947, 12:33.

in its absence. Such organisms may possibly lack certain respiratory enzymes necessary for anaerobic respiration, but there is no proof of this. It is also possible that end products of their anaerobic metabolism are toxic to them, or that they cannot grow in the presence of strong reducing intensities. This group includes such well known bacteria as the nitrifying bacteria, some of the sulfur bacteria, *Bacillus subtilis* and related forms, Azotobacter, the diphtheria bacillus, the cholera vibrio, and others.

However, most bacteria can grow in the complete or virtual absence of molecular oxygen. Two types of anaerobic bacteria may be distinguished. The most numerous are the *facultative anaerobes*, which respire with equal facility in the presence or absence of molecular oxygen. In broth cultures, the dissolved oxygen is soon exhausted and, after a preliminary period of aerobic growth, the culture continues to develop under essentially anaerobic conditions. The *obligate anaerobes* are unable to grow in the presence of molecular oxygen, and oxygen is actively toxic to them; vegetative cells die very quickly upon exposure to air, although spores are highly resistant. Members of the genus Clostridium, together with microaerophilic forms which tolerate very low concentrations of oxygen, are usually included in this group.

Bacteria are unexcelled in the possession of powerful and varied means for obtaining energy from nutrients in the absence of oxygen. The energy obtained from a given quantity of nutrients is usually much less under anaerobic conditions than in the presence of oxygen, because oxidation of substrates is less complete, and anaerobic metabolism is characterized by a rapid utilization of oxidizable substrates and a great accumulation of partially oxidized end products.

Bacteria carry out energy-yielding oxidations under anaerobic conditions in a number of ways. Inorganic compounds may be reduced and thus replace molecular oxygen as the final electron acceptor. For example, ammonia and a single organic compound such as lactic acid are sufficient to support the aerobic growth of *Bact. coli* in a medium of inorganic salts, but growth in the absence of air does not occur unless nitrate ion is also present and the nitrate reduced to nitrite by the enzyme nitratase. Organic compounds may also act as final oxidants in anaerobic respiration. Thus the reduction of fumaric acid to succinic acid by succinic dehydrogenase may replace the reduction of nitrate in supporting the anaerobic growth of *Bact. coli*. Another energy-yielding reaction is a reaction between two substrates in which one is reduced and one is oxidized. This type of reaction is called a dismutation, and is a very common type of anaerobic reaction in bacteria. For instance, acetaldehyde may dismutate to acetic acid and ethanol.

$$2CH_3CHO + H_2O \rightarrow CH_3COOH + CH_3CH_2OH$$

While carbohydrate is the chief energy source for both aerobic and anaerobic heterotrophes, certain of the obligate anaerobes possess only a very limited ability to metabolize carbohydrate, and an important source of energy for these bacteria appears to be the coupled oxidation-reduction of pairs of amino acids. *Cl. sporogenes,* for example, carries out the reaction between alanine and proline at a rapid rate[11] (see accompanying equation).

[11] Stickland: Biochem. J., 1934, 28:1746; 1935, 29:889.

$$\underset{\text{alanine}}{CH_3-\underset{\overset{\displaystyle |}{NH_2}}{CH}-COOH} + 2\ \underset{\text{proline}}{\underset{\overset{\displaystyle NH}{\diagdown\diagup}}{\overset{\displaystyle CH_2-CH_2}{\overset{\displaystyle |\qquad |}{CH_2\quad CH}}}-COOH} + 2\ H_2O \longrightarrow$$

$$\underset{\text{from oxidation of alanine}}{CH_3COOH + CO_2 + NH_2} + \underset{\text{from reduction of proline}}{NH_2-CH_2-CH_2-CH_2-CH_2COOH}$$
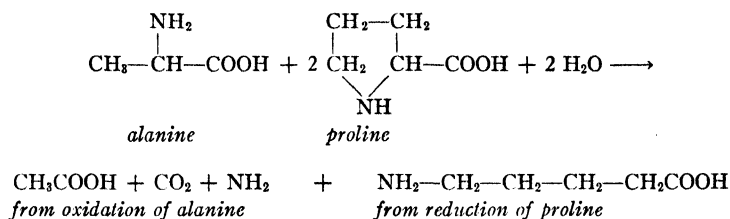
As already mentioned, the obligate anaerobes contain none or very little of the iron-porphyrin respiratory enzymes, a definite indication of enzymatic deficiency. However, the toxicity of molecular oxygen to these bacteria requires more explanation than their lack of oxygen-transporting enzymes. The catalase theory is based on the discovery that certain bacteria produce hydrogen peroxide in amounts sufficient to be toxic to themselves.[12] In cultures of those species which do not produce catalase and are peroxide-sensitive, peroxide accumulates and the culture becomes self-sterilizing; the pneumococcus is an example of this type of microorganisms. Those bacteria which produce catalase decompose the peroxide as it forms and it does not accumulate to toxic concentrations. The obligate anaerobes are peroxide-sensitive and do not form catalase. Hence it has been suggested that these organisms form peroxide rapidly in the presence of air and therefore oxygen is toxic to them. Although this theory is accepted by some, it entirely lacks supporting evidence, for all attempts to demonstrate peroxide in cultures exposed to air have failed.

It has also been proposed that the reducing intensity of the medium is the factor governing whether or not growth will occur. Experimental evidence, both direct and indirect, has been presented which indicates that a certain degree of reducing intensity is essential to the germination of tetanus spores[13]; the positive limit appears to be about $+50$ millivolts at pH 7.0. It has been suggested that this accounts for the failure of tetanus spores to germinate in healthy tissues in the absence of trauma or secondary infection as is sometimes observed. Since a sufficient reducing intensity cannot be maintained in the presence of oxygen, oxygen is toxic. It is not clear why such a reducing intensity should be required. Possibly some of the respiratory enzymes of the obligate anaerobes are oxygen-labile, *i.e.*, active in the reduced state and inactive in the oxidized state, as are some of the bacterial hemolysins (p. 207) or hydrogenase (p. 73), or perhaps the substrates of some of the enzymes of these organisms are unstable under aerobic conditions.

The Role of Phosphorylated Compounds in the Conservation and Utilization of the Energy Liberated in Respiration.[14] Phosphorylated organic compounds are of primary importance in the energy metabolism of bacteria and all other living things. Phosphorylated compounds of biological importance may be conveniently divided into two groups according to the energy liberated upon hydrolysis of the linkage involving the phosphorus.

[12] McLeod and Govenlock: Lancet, 1921, i:900.
[13] Knight and Fildes: Biochem. J., 1930, 24:1496; Quastel and Stephenson: Biochem. J., 1925, 20:1125.
[14] Comprehensive discussions of this subject have been given by Lipmann: Advances in Enzymology, 1941, 1:99; and Kalckar: Chem. Rev., 1941, 28:71.

Low phosphate bond energy compounds	*High phosphate bond energy compounds*
3–4 kg. cal./mole liberated upon hydrolysis of each phosphate bond	10–12 kg. cal./mole liberated upon hydrolysis of each phosphate bond
stable compounds	unstable compounds
strong bond involving phosphorus	weak bond involving phosphorus

All compounds in which the phosphoric acid radical is esterified to an alcoholic hydroxyl have low energy phosphate bonds. Glucose-6-phosphate is a good example of this type of compound:

$$
\begin{array}{c}
H \qquad OH \\
\diagdown \diagup \\
C \\
| \\
HCOH \\
| \\
HOCH \qquad O \\
| \\
HCOH \\
| \\
HC \\
| \\
CH_2-O-P-OH \\
| \\
OH
\end{array}
$$
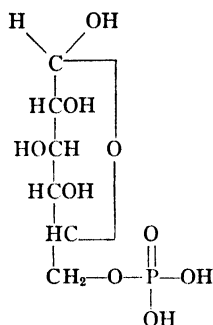
glucose-6-phosphate

High energy phosphate bonds are found in three different kinds of compounds, typified by adenosine triphosphate (ATP), 1, 3-diphosphoglyceric acid and phosphopyruvic acid (see accompanying formulae).

adenosine triphosphate (ATP)
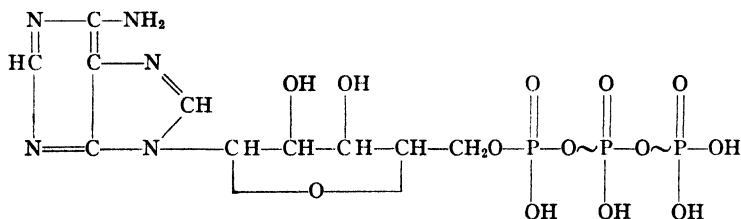
1,3-diphosphoglyceric acid
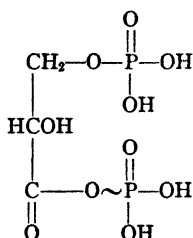
phosphopyruvic acid

(high energy phosphate bond = $\sim$P[15])
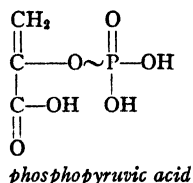
In each of these substances, the high energy phosphate bond results from the formation of an anhydride, *i.e.*, elimination of a molecule of water between

[15] This terminology was introduced by Lipmann (see reference 14).

two compounds. The differences in chemical behavior between compounds with low energy and high energy phosphate bonds are similar to those between ethyl acetate, an ester of acetic acid and ethanol, comparable to low energy phosphorus compounds, and acetyl chloride, an anhydride of acetic and hydrochloric acids, comparable to high energy phosphorus compounds. When water is added to ethyl acetate, no heat (energy) is evolved and the ester is not hydrolyzed. If water is added to acetyl chloride, heat is evolved with explosive violence and acetyl chloride is quickly split into its two component acids. Because of the differences in energy content, compounds with low energy phosphate bonds can never be in reversible equilibrium with compounds containing high energy bonds. Compounds with phosphate bonds of the same energy content may be in equilibrium with each other, and high energy phosphate bonds may be used to form low energy phosphate bonds.

In both the autotrophic and heterotrophic bacteria, the energy released in the oxidation-reduction reactions of respiration may be used to convert inorganic phosphate or the organic phosphate of low bond energy compounds into high phosphate bond energy compounds:

$$\text{substrate-}H_2 + H_3PO_4 + \tfrac{1}{2}\ O_2 \rightarrow \text{substrate} \sim PO_4 + H_2O$$
(or other acceptors)
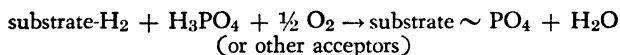
Specific examples of this general process are discussed in other portions of the chapter (*vide infra*).

The adenine nucleotides are of particular importance in the transfer of high energy phosphate bonds from place of generation to place of utilization. The high energy phosphate bonds generated in respiration are transferred to adenosine monophosphate (AMP, adenylic acid) or, as is more often the case, to adenosine diphosphate (ADP) and conserved in the form of ATP.
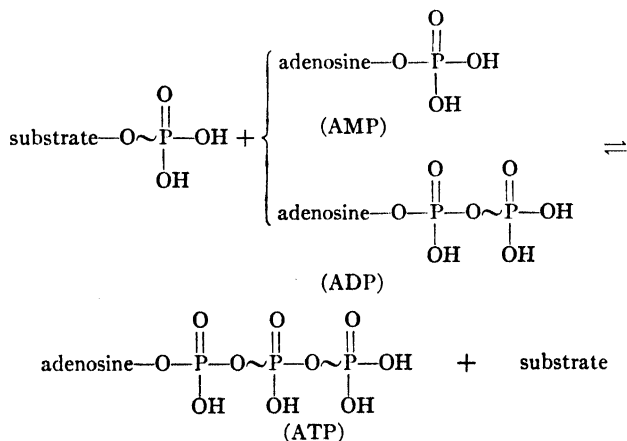
The energy-rich phosphate bonds of ATP are used by bacteria in the assimilation of carbon dioxide, the phosphorylation of carbohydrates and the synthesis of compound sugars (*vide infra*). More generally, it has been demonstrated that ATP is involved in muscular contraction, the synthesis of peptide bonds, the oxidation of fatty acids and many other biochemical processes.

THE AUTOTROPHIC BACTERIA

Of the many types of physiological activity occurring among the bacteria, that of the so-called *autotrophes* is unique in biochemical physiology. The respiratory mechanisms of chemosynthetic organisms are concerned, for the most part, with the oxidation of organic molecules. Such organisms are generally spoken of as *heterotrophes,* organisms which are dependent upon the organic substances synthesized by other living cells. The autotrophic bacteria, however, derive the energy necessary to their life processes from the oxidation of inorganic compounds of nitrogen, sulfur and iron, and obtain nitrogen from ammonium salts and carbon from carbonate or carbon dioxide.

The Nitrifying Bacteria. Of the autotrophic bacteria perhaps the most important single group is that of the microorganisms, discovered by Winogradsky in 1890, which oxidize ammonia to nitrite and nitrite to nitrate. The species of bacteria concerned are Nitrosomonas and Nitrosococcus (so-called because their cultures give the nitroso reaction in the qualitative test for nitrite) which bring about the reaction:

$$NH_3 \longrightarrow NO_2^-$$
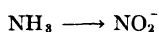
and Nitrobacter which brings about the reaction:

$$NO_2^- \longrightarrow NO_3^-$$
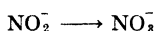
These equations represent the reactions from which these organisms obtain energy for maintenance and growth. In the presence of ammonia and nitrate organic compounds are not oxidized, and their presence may even inhibit growth.[16] However, Boltjes has observed a favorable influence of certain protein derivatives and fatty acids upon the growth of nitrifying bacteria, and Bömeke has shown that, in the absence of ammonia and nitrite, these organisms carry out an oxidation of organic substances, presumably of endogenous origin.

The relative simplicity of the respiratory mechanisms of these two species of nitrifying bacteria has made possible a reliable determination of the free energy efficiency of these organisms. The ratio of nitrogen oxidized to carbon fixed as protoplasm has been found to be 35 for Nitrosomonas and 135 for Nitrobacter. Assuming the energy consumed in the synthesis of protoplasm to approximate that of the reduction of carbon dioxide to hexose, the efficiency of Nitrobacter becomes 7.8 per cent and that of Nitrosomonas 5.9 per cent.[17] Presumably, then, about 95 per cent of the energy liberated should appear as heat, and experiment has shown this to be true. As machines, therefore, the nitrifying bacteria are not highly efficient.

The actual process of oxidation is entirely unknown; it has been suggested that hydroxylamine and hyponitrous acid may be intermediates, but there is no evidence for this.

[16] For example, 0.015 M glucose completely inhibits Nitrobacter in liquid media and somewhat higher concentrations, 0.2 per cent, are necessary to inhibit growth in sand media or soil. (Coleman: Centralbl. f. Bakt., 1908, Abt. II, 20:401, 485.)

[17] Calculated on a free energy rather than heat of combustion basis. *Cf.* Bass-Becking and Parks: *Energy Relations in the Metabolism of Autotrophic Bacteria.* Physiol. Rev., 1927, 7:85.

The organisms may be isolated by the inoculation of mineral salt solutions with soil, the nutriment being carbonate and an ammonium salt in the case of Nitrosomonas and Nitrosococcus and carbonate and nitrite for Nitrobacter. Isolation by the usual pour plate pure culture methods is difficult if not impossible, but colonies may be secured by inoculating silica gel plates from the enrichment cultures. Nitrosomonas grows in microscopic, colorless colonies which turn brown on continued incubation, while Nitrobacter produces somewhat larger colonies, light brown in color.

The oxidation of ammonia is brought about not by a single species of bacterium but rather by a group of closely related organisms. *Nitrosomonas europa*, found in western Europe, is a coccobacillus about 1.5 by 1.0 μ and exists in two forms, one a zooglea of closely packed cells and the other an actively motile swarming (monas) stage in which each cell has a single flagellum. Morphologically similar organisms have been found in this country and elsewhere, although in some cases one stage appears to predominate over the other to such a degree that some might be taken to be different organisms. Coccus forms, given the generic name Nitrosococcus, have also been isolated in various parts of the world. A number of species have been reported which some workers group under the single head of *Nitrosococcus nitrosus*. These organisms are all gram-positive, obligate aerobes. The nitrite oxidizing organisms appear to be somewhat more homogeneous. All are rod-shaped, 1.0 by 0.3 to 0.4 μ in size, with one or both ends pointed, non-motile and gram-negative or gram-positive. Neither Nitrobacter nor the ammonia oxidizing organisms form spores.

The Sulfur Bacteria. The group of autotrophic bacteria that derive their energy from the oxidation of sulfur and its compounds is, both morphologically and physiologically, somewhat more complex than that comprising the nitrifying organisms. Some of the organisms, the filamentous forms, are more closely related to the lower fungi than are the so-called "true bacteria." Beggiatoa, one of the common forms, shows a close resemblance to the blue-green alga Oscillaria. Others, such as Thiobacillus, are morphologically indistinguishable from the usual bacteria. In all, three morphological groups may be observed:

(1) The thread-forming, filamentous forms usually regarded as "higher bacteria" which accumulate sulfur granules within the cells. Three genera are ordinarily included in this group: Beggiatoa, Thiothrix and Thioplaca.

(2) Non thread-forming organisms existing in a number of forms and including such genera as Thiobacillus, Thiospirillum, Thiovulum, Achromatium, etc. Thiobacillus is often separated from the other organisms on the basis of the failure to accumulate sulfur granules within the cells.

(3) The purple and green sulfur bacteria, which differ from the other two groups in that they are pigmented, the pigments making possible a photosynthetic metabolism.

The sulfur bacteria are widely distributed over the earth and are commonly found in the water of sulfur springs, in sewage-laden streams, in swamps where masses of vegetable matter are undergoing slow decomposition and, in fact, wherever sulfur and hydrogen sulfide are present. Whether such organisms bear any relation to the laying down of sulfur deposits is uncertain. It has been suggested that possibly gypsum deposits may have

resulted from the microbial oxidation of sulfur to sulfate neutralized by calcium salts present in the earth.

The Sulfide Bacteria. The oxidation of sulfur compounds by these organisms proceeds through a series of stages. The so-called "sulfide bacteria," including the filamentous forms, derive their energy primarily through the oxidation of hydrogen sulfide to elementary sulfur, which is deposited as granules within the cells:

$$H_2S + \tfrac{1}{2} O_2 \longrightarrow H_2O + S$$
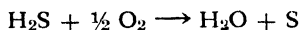
The reaction is the energy-yielding process of these organisms. If the supply of hydrogen sulfide runs low, the organisms further oxidize the accumulated sulfur to sulfate:

$$S + 1\tfrac{1}{2} O_2 + H_2O \longrightarrow H_2SO_4$$
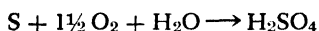
The Thionic Acid Bacteria. A second physiological group, called the thiosulfate or thionic acid bacteria, obtains its energy primarily through the oxidation of thiosulfate, though some members are capable of oxidizing elementary sulfur. The obligate autotrophe, *Thiobacillus thioparus*, is included in this group, together with *Thiobacillus denitrificans*. Some strains of the latter have been found to be obligate autotrophes and also obligate anaerobes, growing in the presence of nitrate, while other strains are both facultative autotrophes and facultative anaerobes. *Thiobacillus thioparus* accumulates free sulfur outside of the bacterial cells when grown in thiosulfate media and the oxidation is supposed to proceed thus:

$$5\ Na_2S_2O_3 + H_2O + 4\ O_2 \longrightarrow 5\ Na_2SO_4 + H_2SO_4 + 4\ S$$
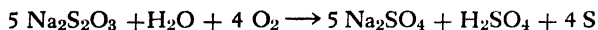
This organism is also able to oxidize sulfide and tetrathionate but oxidizes elementary sulfur to sulfate only very slowly. A number of common enteric bacilli, however, are able to reduce tetrathionate.

The oxidation of sulfur and reduction of nitrate by *Thiobacillus denitrificans* growing under anaerobic conditions proceeds either as:

$$5\ S + 6\ KNO_3 + 2\ H_2O \longrightarrow K_2SO_4 + 4\ KHSO_4 + 3\ N_2$$
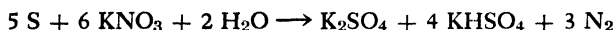
or, in the presence of calcium carbonate, as:

$$5\ S + 6\ KNO_3 + 2\ CaCO_3 \longrightarrow 3\ K_2SO_4 + 2\ CaSO_4 + 2\ CO_2 + 3\ N_2$$

The organism is able to oxidize thiosulfate anaerobically in the presence of nitrate according to the following reaction:

$$5\ Na_2S_2O_3 + 8\ KNO_3 + 2\ NaHCO_3 \longrightarrow 6\ Na_2SO_4 + 4\ K_2SO_4 + 2\ CO_2 + 4\ N_2 + H_2O$$
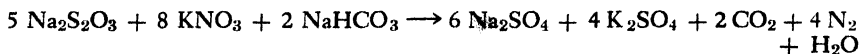
The Sulfur Bacteria. A third group, the sulfur bacteria, is represented by only one species, *Thiobacillus thiooxidans*, which derives its energy primarily through the oxidation of elementary sulfur to sulfate. It is also able to oxidize thiosulfate to sulfate, both oxidations proceeding as indicated above. This strictly autotrophic organism is remarkable for its tolerance of high acidities. Sulfuric acid accumulates in its cultures as a result of the oxidation of sulfur, and normal growth takes place in concentrations as high as 0.25 molar, and even 0.5 molar does not completely inhibit growth. Although a strict auto-

trophe, *Thiobacillus thiooxidans* is not so sensitive to the presence of organic matter as are some other autotrophes. The work of Vogler and of Umbreit has clarified the mechanism whereby the energy released in the oxidation of sulfur to sulfate is used for the assimilation of carbon dioxide into organic compounds. The oxidation of sulfur in the absence of carbon dioxide is accompanied by the uptake of inorganic phosphate and the formation of ATP. If then carbon dioxide is supplied to the bacteria, even in the absence of oxygen, carbon dioxide is rapidly assimilated and inorganic phosphate is liberated. It thus appears that the energy released in the oxidation of sulfur is stored in the energy-rich bonds of ATP, in which form it is used in the fixation of carbon dioxide into organic compounds. This mechanism for energy transfer is the same as that found in heterotrophic bacteria.

In the absence of sulfur, *Thiobacillus thiooxidans* oxidizes an endogenous polysaccharide. LePage and Umbreit[18] have found that this polysaccharide is metabolized via a series of phosphorylated intermediates identical with those occurring in heterotrophes, and O'Kane has established that this bacterium can carry out the autotrophic synthesis of thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine and biotin, all growth factors for heterotrophic bacteria and higher organisms.

It would seem that the metabolism of this autotrophe, and perhaps others as well, might be divided into two phases. In the first, carbon dioxide is fixed and transformed into a few reactive organic compounds by the use of the energy obtained from the oxidation of inorganic substrates or from light (in the case of the photosynthetic forms). This would be a unique and definitive portion of autotrophic metabolism. In the second phase, these few metabolically active substances are used in the synthesis of cellular material by reactions comparable to those occurring in heterotrophes.

Although the influence of light on the metabolism of the purple and green sulfur bacteria had been suspected for some time, it is only in recent years that the photosynthetic character of their physiological activities has been demonstrated through the work of van Niel.[19] The organisms grow under strictly anaerobic conditions in the presence of hydrogen sulfide and carbonate. In the absence of oxidizable sulfur compounds, purple sulfur bacteria can develop in the presence of organic compounds under completely anaerobic conditions. As in plants, photosynthesis in bacteria is now interpreted as an oxidation-reduction process, and may be expressed by the equations:

$$4(H_2O + light \longrightarrow H + OH)$$
$$4 H + CO_2 \longrightarrow (CH_2O) + H_2O$$
$$2(2 OH + H_2A \longrightarrow H_2O + A)$$
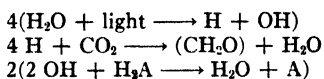
It is to be noted that molecular oxygen is not liberated and the peroxide mechanism of green plants is replaced by one in which hydrogen donators, *e.g.*, hydrogen sulfide, regenerate the system. In the presence of organic matter and the absence of hydrogen sulfide, the reaction is coupled with dehydrogenase

[18] LePage: Arch. Biochem., 1942, *1:*255; see also Umbreit: Bact. Rev., 1947, *11:*157; LePage and Umbreit: Jour. Biol. Chem., 1943, *148:*255; Vogler: Jour. Gen. Physiol., 1942, 25:617.

[19] See the reviews by van Niel: Advances in Enzymology, 1941, *1:*263; Physiol Rev., 1943, 23:338.

systems. It is suggested by van Niel that the photochemical decomposition of water is the light reaction, while the reduction of carbon dioxide is a dark reaction. The green pigment, known as *bacteriochlorin* or *bacteriochlorophyll*, is a pyrrol pigment with a porphin nucleus containing magnesium, and it is closely related to, though not identical with, green plant chlorophylls *a* and *b*.

A group of organisms known as the non-sulfur purple bacteria contain bacteriochlorophyll and are photosynthetic, but differ from the purple sulfur bacteria in that the reduction is coupled only with the oxidation of organic matter, *i.e.*, they are not facultative autotrophes. They are, therefore, intermediate between the sulfur forms and non-photosynthetic bacteria with a typical oxidative metabolism. Available information concerning them has been reviewed by van Niel.[20]

The bacterial oxidation of sulfur and its compounds is of considerable importance in the processes involved in the maintenance of the sulfur cycle. As in the case of nitrogen, many bacteria are capable of reducing sulfur—the evolution of hydrogen sulfide from decomposing organic matter reflects the widespread reduction of sulfur in lieu of oxygen under anaerobic conditions—but only a few are able to oxidize it. These organisms assume considerable practical significance at times, producing corrosion of metals laid in sulfur-containing clay soils[21] and are sometimes responsible for the disintegration of stone and mortar.[22]

The relative simplicity of the respiratory mechanisms of the sulfur bacteria is in itself of no small general physiological interest, but equal importance attaches to the fact that these organisms appear to constitute a link between the autotrophic organisms and those dependent upon preformed organic matter through the species that are facultatively autotrophic. Their efficiency is of the same order as that of the nitrifying bacteria, *i.e.*, 5 to 8 per cent of the energy made available through the oxidation of sulfur and sulfur compounds is utilized in synthesis of cell substances.

The Iron Bacteria. Certain of the filamentous bacteria are characterized by deposits of ferric hydroxide in the sheath surrounding the filament or, sometimes, within the cell itself. It was early suggested that these organisms are able to oxidize ferrous to ferric iron and utilize the energy so liberated. It appears, however, that although some of the so-called iron bacteria are obligate autotrophes which are dependent upon the oxidation of iron as a source of energy, the presence of ferric hydroxide is not necessarily indicative of an autotrophic metabolism. One species, *Spirophyllum ferrugineum*, is an obligate autotrophic organism which grows in inorganic solutions containing ferrous carbonate. The oxidation proceeds according to the following reaction:

$$2 \; FeCO_3 + 3 \; H_2O + \tfrac{1}{2} \; O_2 \longrightarrow Fe_2(OH)_6 + 2 \; CO_2$$
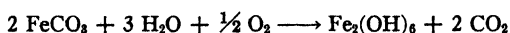
The reaction is exothermic but the energy yield is small, about 15 calories per mol, and large amounts of ferrous salt must be oxidized. Starkey[23] has shown that the oxidation of 55.8 gm. of iron as ferrous carbonate produces 106.8 gm.

[20] van Niel: Bact. Rev., 1944, 8:1.
[21] *Cf.* Bunker: Jour: Soc. Chem. Ind., 1939, 58:93.
[22] Gistl: Centralbl. f. Bakt., Abt. II, Orig., 1940, 102:486.
[23] Starkey: Science, 1945, 102:532.

of ferric hydroxide but only 0.209 gm. of organic cell material, and points out that it is difficult to establish with certainty the reactions whereby the bacteria precipitate the hydroxide. Some of the iron bacteria are also capable of oxidizing manganese salts and precipitate manganese hydrates in their cells.

Other iron bacteria, however, can live without iron and are able to utilize either ferrous carbonate or soluble iron salts of organic compounds. Still others use organic iron compounds but not inorganic iron salts. Harder[24] divides the iron bacteria into three physiological groups:

(1) Those, such as *Spirophyllum ferrugineum,* that precipitate ferric hydroxide from solutions of ferrous carbonate and use the carbon dioxide liberated and the energy produced during the oxidation for their life processes (obligate autotrophes);

(2) Those, such as *Leptothrix ochracea,* that do not require ferrous carbonate but that cause the deposition of ferric hydroxide when either inorganic or organic iron salts are present (facultative autotrophes); and

(3) Those, probably including a number of the lower or ordinary water and soil bacteria, that attack organic iron salts, using the organic acid radical as food and precipitating ferric hydroxide or basic ferric salts, which are gradually changed to ferric hydroxide (heterotrophes).

The best known of the iron bacteria are the Spirophyllum and Leptothrix species indicated above and *Crenothrix polyspora.* These organisms sometimes grow in the conduits of certain public water supplies, where they form unpleasant-looking, brownish, flocculent masses, often leading to complete stoppage of the pipes. The frequent appearance of detached portions of the growth in tap water may give rise to consternation among the water consumers, as in the famous "water calamities" in Berlin, Lille, Rotterdam and other places. There is no evidence that such organisms are directly harmful.

There seems little doubt that iron-depositing bacteria have played an important part in the formation of iron ore deposits, but the relative share of chemical and biological processes in an individual case can be determined only by a thorough study of conditions at the time of deposition, especially as regards sedimentation, climate, depth of water, nature of material in solution and other factors.[24]

The Oxidation of Hydrogen. The bacterial oxidation of hydrogen has already been indicated in the case of the oxidation of hydrogen sulfide to elementary sulfur. Other organisms, given the generic name Hydrogenomonas, are, however, facultative autotrophes which depend upon the oxidation of hydrogen as a source of energy when grown under autotrophic conditions. The hydrogenase is not formed in organic media, but the bacteria grown in inorganic solutions respire with both hydrogen and organic compounds as substrates, suggesting that the hydrogen-oxidizing catalytic system is independent of the normal respiratory process.[25]

The first of these organisms was described in 1906 by Kaserer and named *Hydrogenomonas pantotropha.* Other species were described later, such as *Hydrogenomonas vitrea* and *Hydrogenomonas flava.* The oxidation of hydrogen:

$$H_2 + \tfrac{1}{2} O_2 \longrightarrow H_2O$$
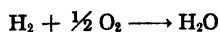
[24] Harder: U. S. Geological Survey, Professional Paper 113, Washington, 1919. See the review by Dorff: Pflanzenforschung, 1934, Heft 16.

[25] Kluyver and Manten: Antonie van Leeuwenhoek Jour. Microbiol. Serol., 1942, 8:71.

yields relatively large amounts of energy (34.2 Cal. per gram as contrasted with 4.1 Cal. per gram of starch) and, although it cannot be accurately measured, the free energy efficiency of these organisms appears to be somewhat higher than that of the other autotrophes, *i.e.*, 10 to 20 per cent.

It has been suggested that all the hydrogen is not burnt directly by these organisms but that a part of it is converted to formaldehyde, which may in turn be either oxidized or used for synthesis.

$$H_2CO_3 + 2\ H_2 \longrightarrow HCHO + 2\ H_2O$$
$$HCHO + O_2 \longrightarrow H_2CO_3$$
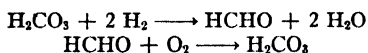
Although most of the hydrogen bacteria are obligate aerobes, some are able to grow anaerobically in the presence of nitrate.

As in the case of the oxidation of iron, the oxidation of hydrogen is not confined to a few species of strict autotrophes, and it has been found that a wide variety of well known heterotrophic bacteria oxidize hydrogen in their respiratory process, as pointed out earlier.

The Oxidation of Carbon Monoxide. The bacterial oxidation of carbon monoxide, comparable in many respects to the oxidation of hydrogen, was reported by Beijerinck and van Delden.[26] These workers isolated an organism which they named *Bacillus oligocarbophilus,* an obligate autotrophe which grew in the absence of organic carbon compounds and obtained its nitrogen from ammonia, nitrite or nitrate. It was later shown that this organism derived its energy through the oxidation of carbon monoxide to carbon dioxide.[27]

$$CO + \tfrac{1}{2}\ O_2 \longrightarrow CO_2$$
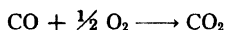
HETEROTROPHIC BACTERIA

It is seldom if ever possible to draw sharp lines of demarcation in biology, and the separation of bacteria into autotrophic and heterotrophic forms being no exception, it must necessarily be to some degree dogmatic. It has already been pointed out that some of the sulfur, iron and hydrogen bacteria are facultative autotrophes, *i.e.*, that they may live by oxidation of either inorganic or organic compounds. Such forms may well be regarded as connecting links between the two physiological types. A somewhat different kind of interconnection is the sharing, between obligate autotrophes and obligate heterotrophes, of common metabolic mechanisms, such as the utilization of gaseous hydrogen, the assimilation of carbon dioxide and the phosphorylation of carbohydrates. Clearly, then, the distinction between the two physiological types is not a sharp one even though the concepts autotrophe and heterotrophe remain extremely useful.

CARBOHYDRATE METABOLISM

Carbohydrate is the chief energy source for almost all heterotrophic bacteria, The functional equivalence of both aerobic and anaerobic metabolism of

[26] Beijerinck and van Delden: Centralbl. f. Bakt., 1903, Abt. II, 2:33.

[27] Distilled water standing in the laboratory for long periods may acquire immunologic properties presumably owing to the growth of bacteria. Organisms able to develop on ammonia and carbon monoxide absorbed from the laboratory air may, in some cases, account for such growth on distilled water, a medium which might be supposed to be entirely lacking in nutriment.

carbohydrate was first recognized by Pasteur, and it is now evident that, although bacteria form many substances from carbohydrate, the breakdown of carbohydrate proceeds in general along well defined pathways, and variations among species are not so great as was once thought. The chief modes of carbohydrate breakdown are summarized in the accompanying diagram.

CARBOHYDRATE BREAKDOWN IN BACTERIA

sugar acids, CO_2, and

other products

O_2

polysaccharides $\underset{H_2O}{\overset{H_2O}{\rightleftarrows}}$ GLUCOSE $\underset{H_2O}{\overset{H_2O}{\rightleftarrows}}$ oligosaccharides

H_2O H_2O

other hexoses   other hexoses

$H_2+CO_2 \rightleftarrows$ formic acid +   oxalacetic acid and other
acetic acid   products

H_2O CO_2

PYRUVIC

H_2 ACID H_2

ethanol $\rightleftarrows$ acetaldehyde $\leftarrow$ $\rightleftarrows$ lactic acid $\rightleftarrows$ propionic acid

$+CO_2$

O_2 + O_2 H_2
                        butyric acid $\rightleftarrows$ butyl alcohol

pyruvic acid

                           acetic acid
                           +

CO_2+H_2O acetylmethylcarbinol   acetic acid $+ CO_2$

$+CO_2$

Breakdown of Oligosaccharides and Polysaccharides. Some Pseudomonas species can rapidly oxidize lactose and maltose to the corresponding aldobionic acids,[28] but most bacteria split compound sugars to their component monosaccharides before they are further metabolized. Oligosaccharides and polysaccharides may be hydrolyzed by the addition of a molecule of water to each glycosidic linkage. The hydrolysis of a compound sugar is an irreversible reaction and proceeds with the liberation of energy, but the energy yield is very small in comparison with that made available through oxidation of the component monosaccharides. Bacteria contain enzymes which are capable of hydrolyzing a wide variety of polysaccharides and oligosaccharides.

Cellulose.[29] Although cellulose is the most widely distributed polysaccharide in nature, the ability to bring about its decomposition is possessed by only a few organisms. The enzyme cellulase is found in a few higher organisms, some snails and a few marine forms, but even the herbivorous animals are dependent upon the presence of cellulose-decomposing microorganisms in the intestine for

[28] Stodala and Lockwood: Jour. Biol. Chem., 1947, *171*:213.
[29] See review by Waksman: Bot. Rev., 1940, *6*:637.

the preliminary decomposition of this polysaccharide.[30] The breakdown of cellulose and other polysaccharides in nature is an essential part of the decomposition of plant residues and the consequent returning to the atmosphere of carbon dioxide removed through the photosynthetic activities of chlorophyll-containing plants. Relatively few species of bacteria are able to decompose cellulose, but those that do are widely distributed in the soil and the mud of sea bottoms as well as in the intestines of herbivorous animals.

A number of bacteria, both aerobic and anaerobic forms, have been isolated which decompose cellulose actively in pure culture. Perhaps the best known aerobic organism is a spiral form, *Spirochaeta cytophaga*, which occurs in soil in considerable numbers. A large number of other bacteria which bring about the aerobic decomposition of cellulose have been studied, but the identity of many of them is doubtful. The breakdown of cellulose by anaerobic bacteria is ordinarily accompanied by further decomposition of the glucose so formed to hydrogen and methane in some cases and to organic acids and carbon dioxide in others. Some species have been described, such as *Bacillus cellulosae dissolvens*, which was isolated from human feces, but in general these organisms are not well known. Equally obscure are the thermophiles which have been isolated from actively fermenting manure heaps. The decomposition of cellulose in nature is largely, if not entirely, a process brought about by mixed cultures of bacteria.

The hydrolysis of cellulose takes place in two stages: first, a breakdown to the disaccharide cellobiose; and second, the hydrolysis of cellobiose to glucose. These reactions are brought about by two separate enzymes, cellulase and cellobiase respectively, and the decomposition, to cellobiose at least, takes place outside the bacterial cell.

Hemicelluloses and Pectins. In addition to cellulose proper, plant tissue contains other polysaccharide material designated as hemicellulose and pectin. On hydrolysis, both yield pentoses, hexoses and uronic acids. Although hemicelluloses are decomposed in nature, largely under the influence of bacteria present in the soil, the chemistry of these compounds is largely unknown and, in consequence, little is known of the processes of their decomposition.[31]

The decomposition of pectin, through the agency of the enzyme pectinase, is brought about by a comparatively small number of species of anaerobic bacteria. The process itself is of considerable commercial importance since these organisms are responsible for the retting of flax and hemp and for the rotting of certain fruits and vegetables. Retting is accomplished by submerging flax and hemp in water and allowing the plant tissue to remain until the dissolution of pectin allows the separation of the fibers.[32] The organisms responsible for the decomposition of pectin are closely related to the group of anaerobic soil forms, the amylobacter group, which includes the butyric acid organisms and others. A number of other bacteria, including Lactobacillus, Aerobacillus, Micrococcus and Enterococcus, decompose pectins.[33] The nature of the process

[30] Hastings: Bact. Rev., 1944, 8:235.
[31] See Waksman: *Humus.* Williams and Wilkins Company, Baltimore. 1936.
[32] Fuller and Norman: Iowa Agr. Expt. Sta. Res. Bull., 1946, 343:893; *ibid.*, 1946, 344:925.
[33] Werch *et al.*: Jour. Inf. Dis., 1942, 70:231.

of decomposition is obscure, though the sugars resulting from hydrolysis are further decomposed to the usual products of sugar oxidation. Few if any of the pectin-decomposing bacteria are able to hydrolyze cellulose also. The combination of the two properties in a single organism would, of course, result in the decomposition of the flax fibers freed by hydrolysis of the pectin.

Starch. Unlike cellulose, starch may be hydrolyzed by a wide variety of living organisms, and the extracellular enzyme diastase, or amylase, is possessed by many bacteria. Like cellulose, however, starch is hydrolyzed in two stages, first to the disaccharide maltose, and second by a further hydrolysis to glucose. The ability of a given organism to hydrolyze starch has acquired an importance equal to sugar fermentations as a differential character in the study of some bacteria.

Miscellaneous Polysaccharides. A wide variety of gums and mucilaginous substances, polymers designated as pentosans, levans, galactans, and the like according to the nature of the products of their hydrolysis, may be decomposed by bacteria. Polysaccharides of bacterial origin, such as pneumococcus polysaccharide, may be decomposed by other bacteria (p. 394), and bacteria have been found which hydrolyze agar, a polysaccharide resistant to the action of the great majority of bacteria.[34]

Polymeric Degradation. Leibowitz and Hestrin[35] have described a mode of compound sugar degradation which is of wide occurrence in bacteria. For example, *B. subtilis* decomposes sucrose with the liberation of free glucose and the formation of levan, a fructose polysaccharide. This method of sugar breakdown has been termed polymeric degradation and involves the synthesis of a new glycosidic bond at the expense of a previously existing one. The reaction appears to be reversible and may be responsible for the breakdown or synthesis (*vide infra*) of sucrose and polysaccharides.

Phosphorylysis. Polysaccharides and oligosaccharides may also be split, not by the addition of water but by the addition of phosphoric acid instead. Such a cleavage is called phosphorylysis and is catalyzed by a group of enzymes called phosphorylases. The sucrose phosphorylase of *Ps. saccharophilia* catalyzes the reaction[36]:

$$\text{sucrose} + H_3PO_4 \rightleftharpoons \text{glucose-1-phosphate} + \text{fructose}$$

In contrast to hydrolysis, phosphorylysis of glycosidic bonds is reversible, and compound sugars may be synthesized by the reverse reaction (*vide infra*).

Anaerobic Breakdown of Glucose and Other Hexoses. In most bacteria the first step in the metabolism of the six-carbon sugars is their cleavage to two three-carbon compounds. This process, referred to as either fermentation or glycolysis, proceeds without the intervention of molecular oxygen, although it occurs in many organisms under both aerobic and anaerobic conditions. Strictly speaking, the term *fermentation* is best used to describe all the metabolic transformations of carbohydrate which occur without direct reaction with molecular oxygen, and the term *glycolysis* is best limited to the breakdown of

[34] See Stanier: Jour. Bact., 1941, 42:527.

[35] For references to original papers, see Barker and Doudoroff: Ann. Rev. Biochem., 1946, 15:497.

[36] See the review by Doudoroff: Fed. Proc., 1945, 4:241.

hexose to triose and its subsequent conversion to pyruvic acid, lactic acid, ethanol, etc.

The first phase of anaerobic glucose breakdown appears to be the same in all organisms: pyruvic acid is formed by means of a phosphorylating glycolysis. Pyruvic acid, therefore, is almost always an intermediate in bacterial fermentations, and variations in the final products of fermentation may usually be accounted for in terms of variation in the fate of pyruvic acid. Present knowledge of the mechanism of the conversion of glucose to pyruvic acid is derived mainly from the work of Meyerhof, Embden, Parnas, Cori, Warburg, and others on yeast and mammalian muscle.[37]

Stripped of intermediate reactions, the conversion of glucose to pyruvic acid proceeds by three main reactions:

(1) Cleavage of hexose to triose

$$
\begin{array}{ccc}
\text{H} \quad \text{OH} & & \text{O} \\
\diagdown\diagup & & \parallel \\
\text{C}\!-\!\!-\!\!-\!\!-\!\!\rceil & & \text{HC} \\
\mid & & \mid \\
\text{HCOH} & & \text{HCOH} \\
\mid & & \mid \\
\text{HOCH} \quad \text{O} \quad\longrightarrow\quad 2 & & \text{CH}_2\text{OH} \\
\mid & & \textit{glyceraldehyde} \\
\text{HCOH} & & \\
\mid & & \\
\text{HC}\!-\!\!-\!\!-\!\!\rfloor & & \\
\mid & & \\
\text{CH}_2\text{OH} & & \\
\textit{glucose} & &
\end{array}
$$
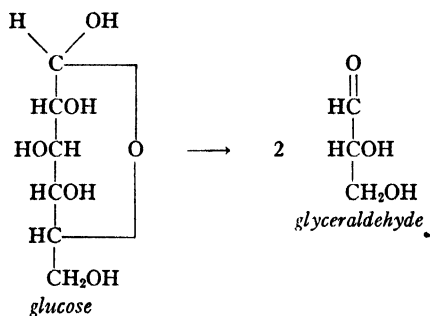
(2) Oxidation of aldehyde to carboxylic acid

$$
\begin{array}{ccc}
\text{O} & & \text{O} \\
\parallel & & \parallel \\
\text{HC} & & \text{COH} \\
\mid & & \mid \\
\text{HCOH} \quad + \text{Acceptor} + \text{H}_2\text{O} \longrightarrow & & \text{HCOH} \quad + \text{Acceptor-H}_2 \\
\mid & & \mid \\
\text{CH}_2\text{OH} & & \text{CH}_2\text{OH} \\
\textit{glyceraldehyde} & & \textit{glyceric acid}
\end{array}
$$
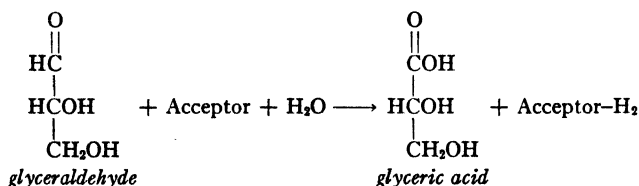
(3) Intermolecular oxidation-reduction

$$
\begin{array}{ccc}
\text{O} & & \text{O} \\
\parallel & & \parallel \\
\text{COH} & & \text{COH} \\
\mid & & \mid \\
\text{HCOH} \quad\longrightarrow & & \text{C}=\text{O} \\
\mid & & \mid \\
\text{CH}_2\text{OH} & & \text{CH}_3 \\
\textit{glyceric acid} & & \textit{pyruvic acid}
\end{array}
$$

The actual process is greatly complicated by the participation of many phosphorylated intermediates and two different coenzyme systems. These complicating factors are important because they serve to make the process almost completely reversible, and they allow the energy liberated in the reaction to be conserved and utilized for other metabolic purposes. The conversion of glucose into pyruvate may be considered to occur in five main stages.

Stage 1. Primary phosphorylation of carbohydrate. Glucose is transformed into glucose-6-phosphate by ATP and the enzyme hexokinase.

$$\text{glucose} + \text{ATP} \longrightarrow \text{glucose-6-phosphate} + \text{ADP}$$

[37] See the reviews of Cori: Biol. Symposia, 1941, 5:131; and Meyerhof: *ibid.*, 1941, 5:141.

The reaction is irreversible. Polysaccharides and oligosaccharides may be hydrolyzed to glucose or other hexoses and phosphorylated in the hexokinase reaction, or they may be split by H_3PO_4 and phosphorylase to form glucose-1-phosphate.

Stage 2. Formation of fructose-1,6-diphosphate. Glucose-1-phosphate is converted into glucose-6-phosphate by the enzyme phosphoglucomutase, which is found in all phosphorylase-containing cells.

$$\text{glucose-1-phosphate} \overset{\text{Mg}}{\rightleftharpoons} \text{glucose-6-phosphate}$$
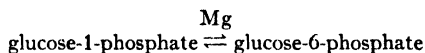
Glucose-6-phosphate, originating either from glucose or from polysaccharide, is then changed into fructose-6-phosphate by the enzyme isomerase.

$$\text{glucose-6-phosphate} \rightleftharpoons \text{fructose-6-phosphate}$$

Fructose-6-phosphate is phosphorylated to fructose-1,6-diphosphate by ATP.

$$\text{fructose-6-phosphate} + \text{ATP} \longrightarrow \text{fructose-1,6-diphosphate} + \text{ADP}$$
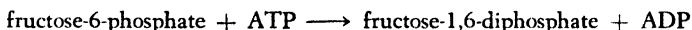
This irreversible reaction is catalyzed by the enzyme phosphohexokinase. Of the other hexoses, fructose and mannose may be converted directly into fructose-1,6-diphosphate. Hexokinase catalyzes the phosphorylation of both glucose and fructose, and a mannose hexokinase appears to be responsible for the fermentation of mannose. However, galactose cannot be converted into fructose-1,6-diphosphate without the inversion of the groups about carbon atom four, yet those organisms which ferment galactose seem to do so by a mechanism similar to that for glucose. The presence of a galactokinase in yeast has been reported recently.[38]

Stage 3. Cleavage of fructose-1,6-diphosphate to triose. Fructose-1,6-diphosphate is split into 3-phosphoglyceraldehyde and dihydroxyacetonephosphate by the enzyme aldolase. The two trioses are reversibly converted into each other by another enzyme, and since dihydroxyacetonephosphate is not oxidized, almost all the fructose-1,6-diphosphate is eventually converted into 3-phosphoglyceraldehyde.

$$\text{fructose-1, 6-diphosphate} \rightleftharpoons$$
$$\text{dihydroxyacetonephosphate} + \text{3-phosphoglyceraldehyde}$$
$$\text{dihydroxyacetonephosphate} \rightleftharpoons \text{3-phosphoglyceraldehyde}$$
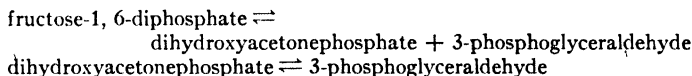
Stage 4. Oxidation of 3-phosphoglyceraldehyde. In the initial stages of glycolysis, before any pyruvic acid has been formed, dihydroxyacetonephosphate is the oxidizing agent for 3-phosphoglyceraldehyde.

$$\text{dihydroxyacetonephosphate} + \text{3-phosphoglyceraldehyde} \rightleftharpoons$$
$$\text{glycerol-1-phosphate} + \text{3-phosphoglyceric acid}$$
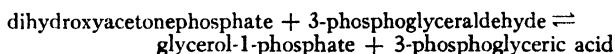
Once pyruvic acid (or a metabolic derivative such as acetaldehyde) is formed, it is reached instead of dihydroxyacetonephosphate, and no more glycerol is formed.

$$\text{pyruvic acid} + \text{3-phosphoglyceraldehyde} \rightleftharpoons$$
$$\text{lactic acid} + \text{3-phosphoglyceric acid}$$
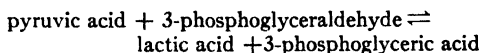
The overall stoichiometric reactions are:

(1) 3-phosphoglyceraldehyde + DPN + H_3PO_4 + ADP $\rightleftharpoons$
     3-phosphoglyceric acid + DPN-H_2 + ATP
(2) pyruvic acid (or acetaldehyde, etc.) + DPN-H_2 $\rightleftharpoons$
     lactic acid + DPN

All components are necessary, and when all are present, the reactions are reversible. Reaction (1) is catalyzed by 3-phosphoglyceraldehyde dehydrogenase, (2) by lactic dehydrogenase. Reaction (1) is actually the summation of three distinct reactions:

[38] Trucco, Caputto, Leloir and Mittelman: Arch. Biochem., 1948, *18*:137.

(1a)   3-phosphoglyceraldehyde + $H_3PO_4 \rightleftharpoons$
                    (H_3PO_4)(3-phosphoglyceraldehyde)

(1b) (H_3PO_4)(3-phosphoglyceraldehyde) + DPN $\rightleftharpoons$
                    1, 3-diphosphoglyceric acid + DPN-H_2

(1c)   1, 3-diphosphoglyceric acid + ADP $\longrightarrow$
                    3-phosphoglyceric acid + ATP
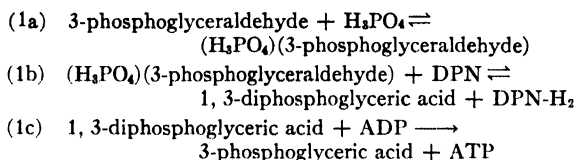
Reactions (1a) and (1b) are catalyzed by 3-phosphoglyceraldehyde dehydrogenase, while reaction (1c) is catalyzed by another specific enzyme. The structure of the primary reaction product between inorganic phosphate and 3-phosphoglyceraldehyde is not known.

The oxidation-reduction reaction of glycolysis is of fundamental importance because it is the best understood example of the coupling of carbohydrate oxidation with the uptake of inorganic phosphate into high energy phosphate bonds. The general reaction may be pictured as:

carbohydrate-H_2 + $H_3PO_4 \rightleftharpoons$ (H_3PO_4) (carbohydrate-H_2)
(H_3PO_4) (carbohydrate-H_2) + Acceptor $\rightleftharpoons$
                    P$\sim$carbohydrate + Acceptor-H_2
P$\sim$carbohydrate + ADP $\longrightarrow$ carbohydrate + ATP
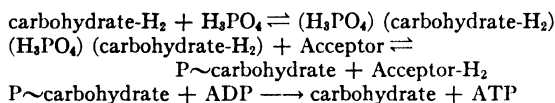
Stage 5. Formation of pyruvic acid. The 3-phosphoglyceric acid formed in the oxidation-reduction is next rearranged into 2-phosphoglyceric acid.

$$\text{3-phosphoglyceric acid} \underset{\text{mutase}}{\overset{\text{triose}}{\rightleftharpoons}} \text{2-phosphoglyceric acid}$$
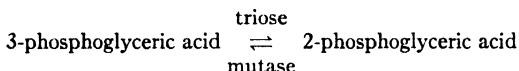
2-Phosphoglyceric acid undergoes an intramolecular oxidation-reduction catalyzed by the Mg-protein enzyme, enolase, to form phosphopyruvic acid.

2-phosphoglyceric acid $\rightleftharpoons$ phosphopyruvic acid
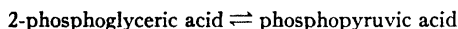
This oxidation is accompanied by the formation of a high energy phosphate bond from a low energy phosphate bond. The high energy phosphate bond of phosphopyruvic acid is transferred to ADP by means of a specific enzyme.

phosphopyruvic acid + ADP $\rightleftharpoons$ pyruvic acid + ATP

The steps by which glucose is broken down to pyruvic acid are summarized in the accompanying diagram.

When one mole of glucose is converted into two moles of pyruvic acid, about 60 kg. cal. of potentially utilizable energy is liberated. In this conversion, two moles of ATP are expended in the formation of fructose-1, 6-diphosphate; and four moles of ATP are generated, two from 1, 3-diphosphoglyceric acid and two from phosphopyruvic acid. This results in a net gain of two moles of ATP, or about 20 kg. cal. The energetic efficiency of the conversion, therefore, is about 33 per cent.

Although the anaerobic breakdown of glucose in bacteria has not been studied in as great detail as the corresponding processes in yeast and muscle, there is strong evidence that the mechanism of conversion of glucose to pyruvic acid is the same.[39]

It has been found that : (1) Inorganic phosphate is taken up into organic linkage during many bacterial fermentations. (2) Phosphorylated hexoses are formed by bacteria. (3) Phosphoglyceric acid is formed from glucose by a large number of bacteria, and when it is added to cultures, normal end products of fermentation are formed. (4) Pyruvic acid is a widely occurring intermediate in the bacterial degradation of glucose. (5) More spe-

[39] Werkman: Bact. Rev., 1939, 3:187.

ANAEROBIC BREAKDOWN OF GLUCOSE TO PYRUVIC ACID

OPO_3H_2

HC————

HCOH

HOCH   O

HCOH

HC————

CH_2OH

glucose-1-phosphate

⇅

OH

HC————

HCOH

HOCH   O

HCOH

HC————

CH_2OH

glucose

$\xrightarrow{ATP}$

OH

HC————

HCOH

HOCH   O

HCOH

HC————

$CH_2OPO_3H_2$

glucose-6-phosphate

⇌

CH_2OH

HOC————

HOC

HCOH   O

HC————

$CH_2OPO_3H_2$

fructose-6-phosphate

$\xrightarrow{ATP}$

$CH_2OPO_3H_2$

HOC————

HOCH

HCOH   O

HC————

$CH_2OPO_3H_2$

fructose-1,6-diphosphate

⇅

CH_2OH

$\overset{|}{C}{=}O$

$CH_2OPO_3H_2$

dihydroxyacetonephosphate

+

HC=O

HCOH

$CH_2OPO_3H_2$

3-phospho-
glyceraldehyde

$\underset{H_3PO_4}{\overset{DPN}{\rightleftharpoons}}$

$\overset{O}{\overset{\|}{C}}OPO_3H_2$

HCOH

$CH_2OPO_3H_2$

1,3-diphospho-
glyceric acid

$\xrightarrow{ADP}$

COOH

HCOH

$CH_2OPO_3H_2$

3-phospho-
glyceric acid

⇌

COOH

$HCOPO_3H_2$

CH_2OH

2-phospho-
glyceric acid

⇌

COOH

$COPO_3H_2$

CH_2

phospho-
pyruvic acid

$\overset{ADP}{\rightleftharpoons}$

COOH

C=O

CH_3

pyruvic
acid

cifically, Utter and Werkman[40] and Still[41] have shown the occurrence of almost all the reactions of the Meyerhof-Embden-Parnas-Cori-Warburg scheme in the fermentations of the coliform bacteria.

While most bacteria convert glucose to pyruvic acid by the same mechanism, they show great diversity in the manner in which they dispose of pyruvic acid once it is formed, thus giving rise to the different types of bacterial fermentations. Under anaerobic conditions, pyruvic acid or one of its derivatives is reduced by accepting electrons, usually from 3-phosphoglyceraldehyde, in a coupled oxidation-reduction reaction.

Lactic Acid Fermentation. The simplest type of fermentation is the lactic acid fermentation in which pyruvic acid itself is the electron acceptor.

$$\text{pyruvic acid} + \text{DPN-H}_2 \rightleftharpoons$$
$$\text{lactic dehydrogenase}$$
$$d\text{-lactic acid} + \text{DPN}$$
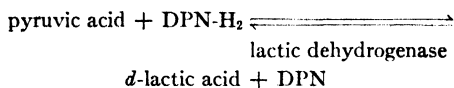
The overall equation for the lactic acid fermentation is:

$$C_6H_{12}O_6 \longrightarrow 2\ C_3H_6O_3$$
$$\textit{glucose} \qquad \textit{lactic acid}$$
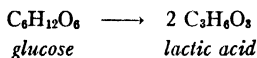
Bacteria of the genera Lactobacillus and Streptococcus form large amounts of lactic acid from glucose, and some species have an almost pure lactic acid fermentation. In addition, lactic acid is one of the products of glucose fermentation in many other bacteria.

Alcoholic Fermentation. In alcoholic fermentation, pyruvic acid is decarboxylated to acetaldehyde by pyruvic carboxylase (in yeast, a Mg-diphosphothiamine-protein).

$$\text{pyruvic acid} \longrightarrow \text{acetaldehyde} + CO_2$$
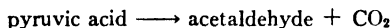
The acetaldehyde then functions as the electron acceptor in the oxidation-reduction reaction of glycolysis and is reduced to ethanol.

$$\text{acetaldehyde} + \text{DPN-H}_2 \rightleftharpoons$$
$$\text{alcohol dehydrogenase}$$
$$\text{ethanol} + \text{DPN}$$
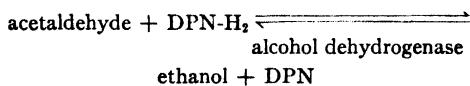
The overall equation for alcoholic fermentation is:

$$C_6H_{12}O_6 \longrightarrow 2\ C_2H_5OH + 2\ CO_2$$
$$\textit{glucose} \qquad \textit{ethanol}$$
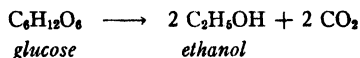
Some Sarcina species carry out an almost pure alcoholic fermentation, and many other bacteria produce small amounts of ethanol from glucose.

Propionic Acid Fermentation. The propionic acid bacteria, which are very closely related to Lactobacillus, reduce pyruvic acid all the way to propionic acid. They also decarboxylate pyruvic acid to acetaldehyde and CO_2.

$$\overset{H_2}{\qquad} \qquad \overset{H_2}{\qquad}$$
$$CH_3CHO + CO_2 \leftarrow CH_3COCOOH \rightleftharpoons CH_3CHOHCOOH \rightleftharpoons CH_3CH_2COOH$$
$$\textit{acetaldehyde} \qquad \textit{pyruvic acid} \qquad \textit{lactic acid} \qquad \textit{propionic acid}$$
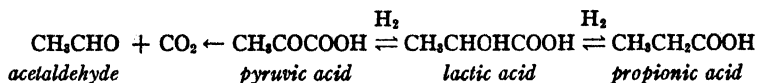
[40] Utter and Werkman: Jour. Bact., 1941, 42:665; Biochem. Jour., 1942, 36:485.
[41] Still: Biochem. Jour., 1940, 34:1177, 1374.

Fermentation of the Coliform Bacteria.[42] The coliform bacteria carry out more complex fermentations of glucose than those just described. The details

FERMENTATIONS OF THE COLIFORM BACTERIA

$$H_3PO_4 + CH_3COOH \longleftarrow CH_3\overset{O}{\overset{||}{C}}O\sim PO_3H_2 + HCOOH \rightleftharpoons H_2 + CO_2$$

   acetic acid   *acetylphosphate*   *formic*
                acid

$$\Uparrow H_3PO_4$$

$$\overset{H_2}{CH_3CH_2OH} \rightleftharpoons CH_3CHO + CO_2 \longleftarrow CH_3COCOOH \underset{H_2}{\rightleftharpoons} CH_3CHOHCOOH$$

  ethanol   *acetaldehyde*   *pyruvic acid*   *lactic acid*

$$+$$

$$CH_3COCOOH$$

$$\downarrow$$

$$\underset{\text{diacetyl}}{CH_3COCOCH_3} \overset{H_2}{\dashrightarrow} \underset{\text{acetylmethylcarbinol}}{CH_3COCHOHCH_3} \overset{H_2}{\rightleftharpoons} \underset{\text{2,3-butylenediol}}{CH_3CHOHCHOHCH_3}$$
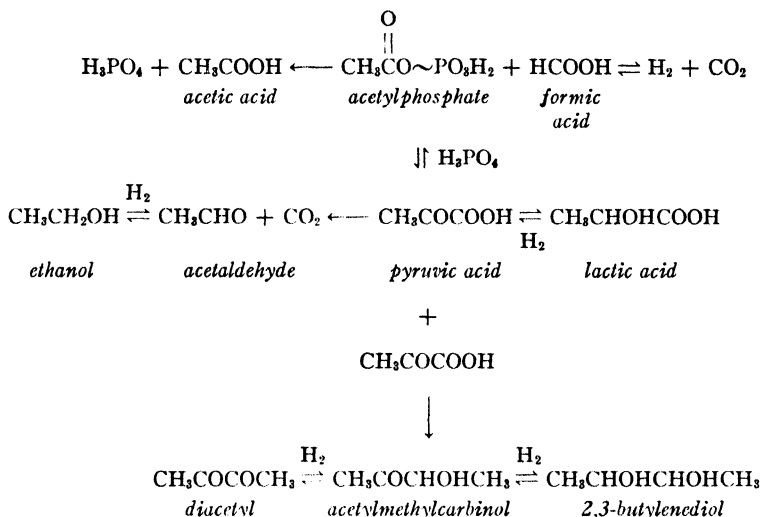
of these fermentations have been elucidated with the aid of cell-free bacterial extracts and isotopic techniques. The accompanying diagram illustrates the manner in which the coliform bacteria, as a group, metabolize pyruvic acid under anaerobic conditions; not all of these reactions occur in any one species. The proportion of fermentation products formed varies with the species, experimental conditions, etc., but a glucose fermentation of *Bact. coli* may be represented by the equation:

$$2 \text{ glucose} + H_2O \longrightarrow 2 \text{ lactic acid} + \text{acetic acid} + \text{ethanol} + 2 CO_2 + 2 H_2$$
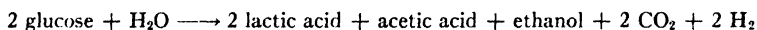
Acetylmethylcarbinol (acetoin) and related substances are formed only by *Bact. aerogenes,* and this fact is of value in the separation of *Bact. aerogenes* from *Bact. coli.* The enzyme which converts pyruvic acid into acetylmethylcarbinol is a Mg- or Mn-diphosphothiamine-protein complex.[43]

Butyl Alcohol Fermentations.[44] Another type of complex bacterial fermentation is carried out by anaerobic organisms such as *Clostridium butylicum, Cl. acetobutylicum, Cl. welchii* and *Cl. kluyverii.* Pyruvic acid is transformed into acetic acid which condenses with itself to form acetoacetic acid (acetylphosphate may be an intermediate). Butyric acid and butyl alcohol are then formed by reduction of the acetoacetic acid. In the anaerobic breakdown of glucose by *Cl. butylicum,* fermentation products are formed in approximately the following molar proportions: 50% CO_2, 25% H_2, 12% butyl alcohol, 3% each of isopropyl alcohol, butyric acid and acetic acid, and traces of ethanol.

[42] For original references, consult Barker and Doudoroff: Ann. Rev. Biochem., 1946, 15:497.

[43] Silverman and Werkman: Jour. Biol. Chem., 1941, 138:35.

[44] For discussion of butyl alcohol fermentations, see Barker and Doudoroff: Ann. Rev. Biochem., 1946, 15:483.

THE BUTYL ALCOHOL FERMENTATION

$$CH_3CHOHCOOH \underset{H_2}{\rightleftharpoons} CH_3COCOOH \underset{H_3PO_4}{\rightleftharpoons} CH_3CO{\sim}PO_3H_2 + H_2 + CO_2$$

lactic acid   *pyruvic acid*   *acetylphosphate*

$$CO_2 + CH_3COCH_3 \leftarrow CH_3COCH_2COOH \rightleftharpoons CH_3COOH + CH_3COOH \rightleftharpoons CH_3CHO \rightleftharpoons CH_3CH_2OH$$

acetone   *acetoacetic acid*   *acetic acid*   *acetal-*   *ethanol*
                                                  dehyde

    $\Updownarrow H_2$ $\Updownarrow 2\,H_2$

$CH_3CHOHCH_3$ $CH_3CH_2CH_2COOH$

isopropyl alcohol   *butyric acid*

               $\Updownarrow 2\,H_2$

            $CH_3CH_2CH_2CH_2OH$

               butyl alcohol
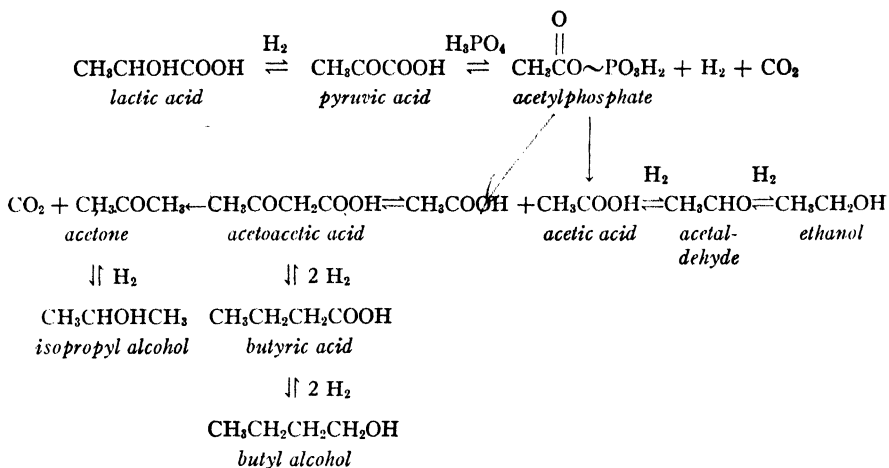
In both of the two complex fermentations described in the preceding paragraphs, pyruvic acid is split to acetic acid, H_2, and CO_2 by addition of a molecule of water. This reaction is known as the hydroclastic reaction:

$$CH_3COCOOH + H_2O \longrightarrow CH_3COOH + H_2 + CO_2$$

pyruvic acid   *acetic acid*
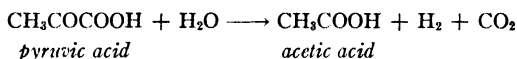
However, recent investigations have revealed that the hydroclastic reaction is actually a reversible phosphoryclastic reaction in which acetylphosphate, which contains a high energy phosphate bond, is formed. In *Bact. coli,* the reaction proceeds in two steps:

$$CH_3COCOOH + H_3PO_4 \rightleftharpoons CH_3C{-}O{\sim}\overset{O}{\underset{OH}{P}}{-}OH + HCOOH$$

pyruvic acid   *formic acid*

                acetylphosphate

               hydrogenylase

$$HCOOH \rightleftharpoons H_2 + CO_2$$
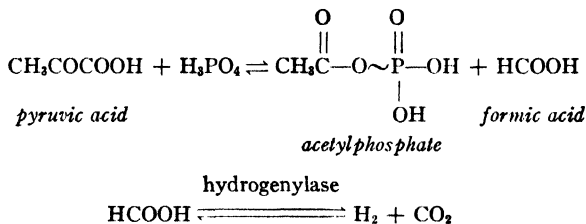
In *Cl. butylicum,* formic acid is not an intermediate in the formation of H_2 and CO_2.

$$CH_3COCOOH + H_3PO_4 \rightleftharpoons CH_3C{-}O{\sim}\overset{O}{\underset{OH}{P}}{-}OH + H_2 + CO_2$$

pyruvic acid   *acetylphosphate*
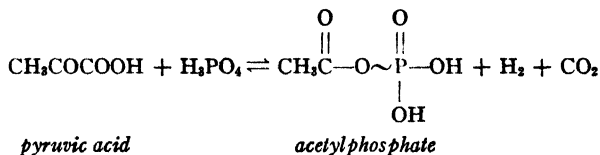
Fermentation of Other Sugars, Organic Acids and Alcohols. The fermentation of sugars other than hexoses, organic acids and alcohols results in the formation of acids and, sometimes, gases; but, for the most part, the mechanisms involved in these decompositions are unknown. Certain fragmentary ob-

servations are available, such as those of Kay,[45] who showed that when mannitol, glucose, glucuronic acid, glycuronic acid and saccharic acid are fermented by *Bact. coli,* the yields of the more oxidized products (acetic and succinic acids) rise and those of the reduced products (ethanol) fall as one proceeds from the reduced substrate mannitol to the more highly oxidized saccharic acid. The fermentation of glycerol by this same organism has been found[46] to yield much the same end products as the fermentation of glucose. Similarly, the fermentation of the four-carbon acids, malic, tartaric and fumaric acids, by *Bact. aerogenes* gives rise to the same products as the glucose fermentation[47]; the fermentation apparently proceeds via oxalacetic acid, which is decarboxylated to pyruvic acid (p. 85).

Energy Yields from Anaerobic Breakdown of Pyruvic Acid. Bacterial fermentation of pyruvic acid always results in the liberation of energy, but the diverse nature of the products of fermentation makes exact calculation of the energy changes very difficult. Under proper conditions (see p. 115), many heterotrophic bacteria, such as *Bact. coli,* may grow anaerobically with only pyruvic acid, lactic acid or glycerol as the source of energy. The formation of acetylphosphate and the transfer of its high energy phosphate bond to the adenine nucleotides is of frequent occurrence in the bacterial fermentations and represents one possible mechanism of energy conservation and transfer.

Aerobic Breakdown of Carbohydrate. Comparatively little attention has been paid to the aerobic metabolism of carbohydrate in bacteria, and the details of these processes are not well understood. Certain bacteria are able to oxidize such diverse compounds as methane, benzene, phenol, cholesterol, etc.,[48] but the substrates for most aerobic bacterial oxidations are the hexoses and their anaerobic fermentation products.

Hexoses may be oxidized without preliminary fermentation. Some bacteria which are unable to ferment glucose can oxidize glucose or glucose-6-phosphate with molecular oxygen.[49] The acetic acid bacteria are noted for the variety of direct oxidation products which they form. These bacteria can oxidize glucose to gluconic acid, 5-ketogluconic acid, and 2-ketogluconic acid.

Direct Oxidation of Pyruvic Acid. Enzymes which catalyze the oxidation of pyruvic acid to acetic acid and CO_2 are formed by many bacteria. Like the enzymes which decarboxylate pyruvic acid to acetaldehyde and CO_2 or convert it to acetylmethylcarbinol and CO_2, the pyruvic oxidases are diphosphothiamine-protein complexes.

diphosphothiamine
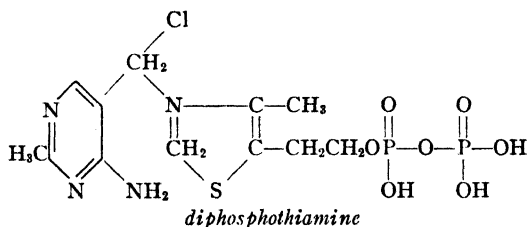
[45] Kay: Biochem. Jour., 1926, 20:231.
[46] Braak: Diss., Delft, 1928.
[47] Barker: Kon. Akad. Weten. Amsterdam, 1936, 39:1.
[48] *Cf.* the review by Zobell: Bact. Rev., 1946, 10:1.
[49] Barron and Friedemann: Jour. Biol. Chem., 1941, 137:593.

The exact function of diphosphothiamine, or cocarboxylase, in the metabolism of pyruvic acid is not known, and the reversible oxidation-reduction of this coenzyme has not been demonstrated.

In *Bact. coli*[50] and the gonococcus,[51] pyruvic acid is oxidized in a reaction in which diphosphothiamine, but not inorganic phosphate, is required, and electron transport involves the flavoproteins and the cytochrome system. In *Proteus vulgaris*[52] the oxidation of pyruvic acid also proceeds without uptake of phosphate, but the pyruvic oxidase of this bacterium is a Mg-diphosphothiamine protein which apparently reacts directly with oxygen. In these types of pyruvic acid oxidation, the equation for the reaction is:

$$CH_3COCOOH + \tfrac{1}{2} O_2 \longrightarrow CH_3COOH + CO_2$$
$$\textit{pyruvic acid} \qquad\qquad \textit{acetic acid}$$
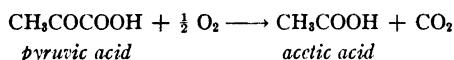
Lipmann[53] has described a third type of pyruvic acid oxidation in *Lactobacillus delbrueckii* in which the oxidation is coupled with the uptake of inorganic phosphate and acetylphosphate is formed.

$$CH_3COCOOH + H_3PO_4 + O_2 \longrightarrow CH_3CO{\sim}PO_3H_2 + CO_2 + H_2O_2$$
$$\textit{pyruvic acid} \qquad\qquad\qquad\qquad \textit{acetylphosphate}$$
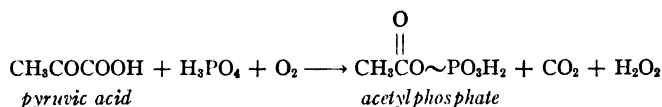
The pyruvic oxidase of *L. delbrueckii* is a Mg-diphosphothiamine protein, and oxygen uptake is due to the presence of an autooxidizable protein (p. 71).

Anaerobic breakdown products of pyruvic acid are also oxidized by molecular oxygen. Ethanol is oxidized to acetic acid by the bacteria responsible for the vinegar "fermentation"; the propionic acid bacteria oxidize acetaldehyde to acetic acid; and acetic acid itself may be oxidized to CO_2 and H_2O.

Oxidation of the Four-Carbon Dicarboxylic Acids. Many bacteria rapidly oxidize the four-carbon dicarboxylic acids. Anaerobically, the reverse reactions occur, and oxalacetic acid may be reduced to succinic acid.

$$\begin{array}{ccccc}
& \tfrac{1}{2} O_2 & & H_2O & \\
\text{succinic acid} & \longrightarrow & \text{fumaric acid} & \longrightarrow & \\
& \text{succinic} & & \text{fumarase} & \\
& \text{dehydrogenase} & & &
\end{array}$$

$$\begin{array}{ccc}
& \tfrac{1}{2} O_2 & \\
\text{malic acid} & \longrightarrow & \text{oxalacetic acid} \\
& \text{malic} & \\
& \text{dehydrogenase} &
\end{array}$$
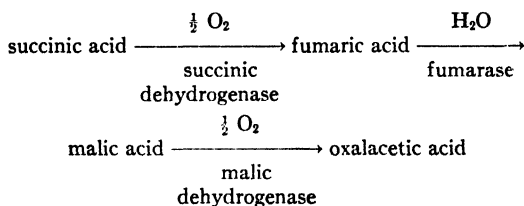
Szent-Györgyi and Krebs have shown that the oxidation of carbohydrates in higher animals is catalyzed by the four-carbon dicarboxylic acids, and such catalysis also occurs in bacteria. For example, in the oxidation of glucose by *Micrococcus lysodeikticus,* small amounts of fumaric acid cause an increased

[50] Still: Biochem. Jour., 1941, 35:380.
[51] Barron and Lyman: Jour. Biol. Chem., 1939, 127:143.
[52] Stumpf: Jour. Biol. Chem. 1945, 159:529.
[53] Lipmann: Cold Spring Harbor Symposium on Quantitative Biology. 1939, 7:248; Jour. Biol. Chem., 1944, 155:55.

THE KREBS TRICARBOXYLIC ACID CYCLE

COOH
C=O
CH$_3$
*pyruvic
acid*

+

COOH
C=O
CH$_2$
COOH
*oxalacetic
acid*

DPN
½ O$_2$

½ O$_2$

H$_2$O

COOH
CH$_2$
HO C COOH
CH$_2$
COOH
*citric
acid*

COOH
CHOH
CH$_2$
COOH
*malic
acid*

H$_2$O

CO$_2$ +

COOH
CH
C COOH
CH$_2$
COOH
cis-*aconitic
acid*

H$_2$O

COOH
CH
‖
CH
COOH
*fumaric
acid*

½ O$_2$

COOH
CHOH
H C COOH
CH$_2$
COOH
*isocitric
acid*

TPN
½ O$_2$

COOH
CH$_2$
CH$_2$
COOH
*succinic
acid*

+CO$_2$

½ O$_2$

COOH
C=O
CHCOOH
CH$_2$
COOH
*oxalosuccinic
acid*

COOH
C=O
CH$_2$
CH$_2$
COOH
*α-ketoglutaric
acid*

+CO$_2$

oxygen uptake five times greater than the amount of oxygen required for the complete oxidation of the added fumaric acid to CO_2 and H_2O.[54]

The Szent-Györgyi Cycle.[55] According to Szent-Györgyi such catalysis occurs because fumaric and oxalacetic acids act as hydrogen acceptors in the oxidation of glucose and are reduced to succinic acid which is reoxidized by molecular oxygen. This series of reactions is referred to as the Szent-Györgyi cycle. Many bacteria aerobically oxidize succinic acid faster than glucose itself, and the rate of anaerobic glucose oxidation in the presence of excess fumaric acid may be as great as the rate of aerobic glucose oxidation. Thus, it is possible for the Szent-Györgyi cycle to function in the respiration of some bacteria, but its actual occurrence has not been observed.

The Krebs Cycle.[55] A considerable portion of the carbohydrate metabolized by most aerobic organisms is oxidized completely to CO_2 and H_2O, usually by way of pyruvic acid as an intermediate. In a wide variety of organisms, the complete oxidation of pyruvic acid is brought about, not by a single reaction, but by a cyclic series of reactions, the tricarboxylic acid cycle of Krebs. In this scheme, pyruvic acid condenses with oxalacetic acid to form a six-carbon tricarboxylic acid which is oxidized and decarboxylated in a series of reactions ending in the regeneration of oxalacetic acid. The net reaction of the cycle is:

$$C_3H_4O_3 + 2\tfrac{1}{2}O_2 \longrightarrow 3\ CO_2 + 2\ H_2O$$
pyruvic acid
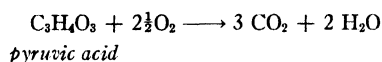
The tricarboxylic acid cycle has been demonstrated in mammalian and avian tissues and in protozoa. It probably also functions in plants and fungi. While almost all of the reactions of the tricarboxylic acid cycle occur in bacteria, it has not been shown that the cycle functions in the oxidation of pyruvic acid by bacteria. The chief difficulty in demonstrating the Krebs cycle in bacteria lies in the inability of most bacteria, *Bact. coli* for example, to oxidize citric, isocitric or *cis*-aconitic acid. Those bacteria which can oxidize the tricarboxylic acids (*Bact. aerogenes*, etc.) apparently form only succinic acid, acetic acid and CO_2, not α-ketoglutaric acid as required by the tricarboxylic acid cycle. However, Werkman[54] believes that some sort of a tricarboxylic acid cycle may be operative in bacteria.

Energy Yield from Aerobic Oxidation of Carbohydrates. The yield of useful energy from complete aerobic oxidation of glucose to CO_2 and H_2O is much greater than the yield from anaerobic conversion of glucose to lactic acid, ethanol, etc.

glucose $\longrightarrow$ 2 lactic acid   54 kg. cal./mole energy liberated
2 lactic acid + 6 O_2 $\longrightarrow$
        6 CO_2 + 6 H_2O 632 kg. cal./mole energy liberated
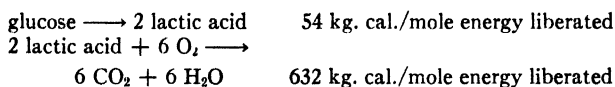
In the tissues of higher animals, glucose and other carbohydrates are oxidized

[54] Werkman: in *A Symposium on Respiratory Enzymes.* University of Wisconsin Press, Madison. 1942. p. 258.

[55] For evaluation of the role of the Szent-Györgyi cycle and the tricarboxylic acid cycle in bacterial metabolism, see Krebs: Advances in Enzymology, 1943, 3:191, and reference 54.

almost completely to CO_2 and H_2O under aerobic conditions. In these tissues, the energy liberated in the oxidation of carbohydrate is conserved with great efficiency in high energy phosphate bonds. As many as fifteen such bonds may be generated by the oxidation of a single mole of pyruvic acid.[56] This represents about the same energetic efficiency as obtained in the anaerobic oxidation of glucose. When bacteria oxidize carbohydrate completely to CO_2 and H_2O, it is probable that a similar formation of high energy phosphate bonds occurs, but it has not yet been experimentally demonstrated. When aerobic oxidation of carbohydrate is incomplete, as in many bacterial oxidations, the energy change is not nearly so great. For instance, the utilizable energy released in the oxidation of pyruvic acid to acetic acid and CO_2 is only sufficient to generate one energy-rich phosphate bond.

Synthesis of Cell Substances from Carbohydrates. The breakdown of carbohydrates by the series of energy-yielding reactions just discussed is actually only one of several ways in which carbohydrates are utilized by the bacterial cell. They may also be combined with CO_2 to form new substances, converted into oligosaccharides and polysaccharides, or transformed into non-carbohydrate cell substances. These are the processes by which bacteria change the carbohydrates of the culture medium into their own highly specific cellular components. Such reactions generally do not proceed spontaneously because they require an external source of energy. The energy-requiring reactions may be designated as synthetic reactions in contrast to the energy-yielding degradative reactions. In the living cell, the two types of reactions occur in such a manner that the energy released in the breakdown of carbohydrates is used in part for the synthesis of other carbohydrates, usually in the form of high energy phosphate bonds.

Assimilation of Carbon Dioxide.[57] It has been known for many years that CO_2 is involved in the metabolism of heterotrophic bacteria, because it had been repeatedly observed that many bacteria did not grow when all CO_2 was removed from the culture medium with CO_2-free air. However, proper interpretation of these findings did not come until 1935, when Wood and Werkman discovered that the heterotrophic propionic acid bacteria fix carbon dioxide into organic linkage, mainly in the carboxyl groups of the four-carbon dicarboxylic acids. Later investigations employing isotopically labelled CO_2 confirmed this original observation and demonstrated the occurrence of CO_2 fixation in other bacteria, yeasts, fungi, protozoa and higher animals.

Several different primary fixation reactions are responsible for the assimilation of CO_2 in heterotrophic bacteria. They may be classified on the basis of the substance which reacts with CO_2 (the specified organism is that in which the reaction was first reported):

(1) No C–C linkage
   in *Methanobacterium omelianskii*
   $CO_2 + 4$ Acceptor–$H_2 \longrightarrow CH_4 + 4$ Acceptor $+ 2 H_2O$
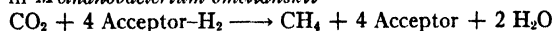
[56] Ochoa: Jour. Biol. Chem., 1943, *151*:493.

[57] This subject has been reviewed by Werkman and Wood: Advances in Enzymology, 1942, 2:135; Krebs: Ann. Rev. Biochem., 1943, *12*:529; and Wood: Physiol. Rev., 1946, 26:198.

(2) C_1–C_1 addition

    in *Cl. acidiurici*

    $2\ CO_2 + 4\ \text{Acceptor–}H_2 \longrightarrow 2\ CH_3COOH + 4\ \text{Acceptor} + 2\ H_2O$

(3) C_2–C_1 addition

    a. in *Cl. butylicum*

    $CH_3COPO_3H_2 + H_2 + CO_2 \rightleftharpoons CH_3COCOOH + H_3PO_4$

    b. in *Bact. coli*

    $H_2 + CO_2 \rightleftharpoons HCOOH$

    $HCOOH + CH_3COPO_3H_2 \rightleftharpoons CH_3COCOOH + H_3PO_4$

(4) C_3–C_1 addition

    in *Propionibacterium pentosaceum*

$$
\begin{array}{lcl}
\text{COOH} & & \text{COOH} \\
| & & | \\
\text{C=O} + CO_2 & \rightleftharpoons & \text{C=O} \\
| & & | \\
\text{CH}_3 & & \text{CH}_2 \\
& & | \\
& & \text{COOH}
\end{array}
$$

Of these modes of addition, the carboxylation of pyruvic acid to oxalacetic acid, the Wood-Werkman reaction, is the most widely distributed. An additional primary fixation reaction, the addition of CO_2 to a-ketoglutaric acid to form oxalosuccinic acid, occurs in animal tissues but has not yet been found in bacteria.[58] Reductive fixation of CO_2 (fixation reactions 1 and 2) is accompanied by the oxidation of a hydrogen donor, which may be any of several simple organic compounds, such as ethanol. In non-reductive fixation (fixation reactions 3 and 4) the CO_2 appears in the carboxyl group of an organic acid without being reduced. After CO_2 has been incorporated into an organic compound by one of the primary fixation reactions, further metabolic reactions may lead to the distribution of the fixed CO_2 among many different substances. Thus, Wood and Werkman found that the CO_2 fixed in oxalacetic acid by the propionic acid bacteria also appeared in the other four-carbon dicarboxylic acids and in propionic acid.

The ability to fix CO_2 into organic linkage was once thought to be the exclusive property of autotrophic organisms, but after demonstration of CO_2 fixation in the heterotrophic propionic acid bacteria, the line between autotrophes and heterotrophes was redrawn by assuming that only autotrophes can join two molecules of CO_2 together in organic linkage. However, when it was found that *Cl. acidiurici* can synthesize acetic acid from two molecules of CO_2, even this difference between the two metabolic types was destroyed. The distinction between autotrophic and heterotrophic CO_2 fixation seems to be only quantitative: heterotrophes derive only a fraction of their carbon compounds from CO_2, while autotrophes obtain all of their compounds by assimilation of CO_2.

Studies of the energetics of CO_2 assimilation have emphasized the close similarity of autotrophic and heterotrophic mechanisms. Incorporation of CO_2 into an organic compound requires the expenditure of energy. In the autotrophe, *Thiobacillus thiooxidans*, CO_2 is fixed with energy gained from the oxidation of sulfur to sulfate, and ATP takes part in the transfer of energy from the sulfur-oxidizing to the CO_2-fixing system (p. 81). In a comparable

[58] Ochoa: Jour. Biol. Chem., 1948, 174:133.

fashion, energy for the reductive fixation of CO_2 by *Methanobacterium omelianskii* and *Cl. acidiurici* is obtained from the simultaneous oxidation of organic substrates. In the carboxylation of acetylphosphate to pyruvic acid in *Bact. coli* and *Cl. butylicum*, the high energy phosphate bond of acetylphosphate is probably the source of energy used in fixation, since pyruvic acid is not formed when acetylphosphate is replaced by acetic acid. Carboxylation of pyruvic acid to oxalacetic acid in the Wood-Werkman reaction is catalyzed by the enzyme oxalacetate carboxylase, which has been found both in bacteria and in animal tissues.

$$
\begin{array}{lll}
\text{COOH} & & \text{COOH} \\
| & \quad\quad \text{Mn}^{++} & | \\
\text{C}{=}\text{O} \;+\; {}^*\text{CO}_2 \xrightleftharpoons{\quad\quad} & & \text{C}{=}\text{O} \\
| & \text{oxalacetate} & | \\
\text{CH}_3 & \text{carboxylase} & \text{CH}_2 \\
\textit{pyruvic acid} & & | \\
& & {}^*\text{COOH} \\
& & \textit{oxalacetic acid}
\end{array}
$$
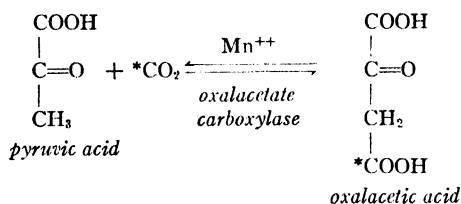
The energy change in the reaction is such that the equilibrium of the reaction is far to the left in favor of decarboxylation of oxalacetic acid. However, when ATP is added to pyruvic acid and CO_2 in the presence of Mn^{++} and oxalacetate carboxylase, oxalacetic acid is formed in amounts detectable by isotopic tracer techniques. The function of ATP in this reaction is unknown.

Assimilation of CO_2 in autotrophes is the source of all organic compounds synthesized by these organisms, but in heterotrophes the function of CO_2 fixation is not so clear. However, from the relatively few direct products of CO_2 fixation, an almost unlimited number of compounds may be produced in further metabolic reactions, and it has been suggested that CO_2 fixation functions in the synthesis of essential metabolites, notably the four-carbon dicarboxylic acids and certain amino acids.

Synthesis of Oligosaccharides and Polysaccharides. The formation of the glycosidic bond is the essential reaction in these syntheses.

semi-acetal hydroxyl
(here, carbon 1 of
an aldohexose)

alcoholic
hydroxyl
(here, carbon
6 of a hexose)
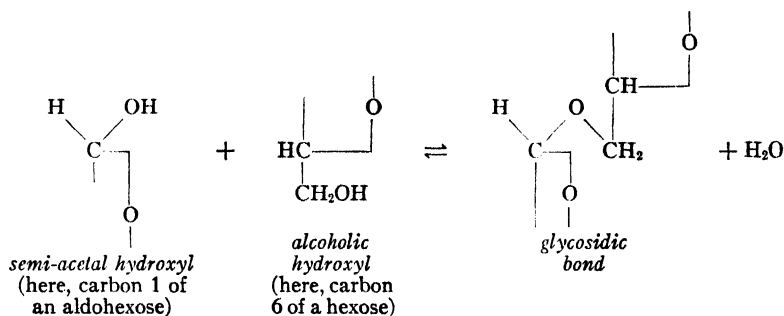
glycosidic
bond

Since the glycosidic bond has a higher energy content than the products of its hydrolysis, the equilibrium of the reaction is in favor of hydrolysis. Therefore, an external source of energy must be used to drive the reaction in the direction of synthesis of the glycosidic link. In bacteria, there are at least two mecha-

nisms for synthesis of polysaccharides and oligosaccharides, both of which meet the energy requirement with the high energy phosphate bonds of ATP.

If the semi-acetal hydroxyl is converted into a phosphate ester, glucose-1-phosphate, the glycosidic bond is easily formed because the phosphate ester bond has about the same energy content as the glycosidic bond itself. Thus, sucrose may be formed in *Pseudomonas saccharophilia* and *Leuconostoc mesenteroides* by the reaction.[36]:

$$\text{glucose-1-phosphate} + \text{fructose} \xrightleftharpoons[\text{phosphorylase}]{\text{sucrose}} \text{sucrose} + H_3PO_4$$
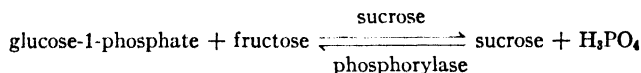
Cori[59] has carried out the *in vitro* synthesis of starch and glycogen by similar reactions catalyzed by phosphorylases from plant and animal sources.

$$n\ (\text{glucose-1-phosphate}) \xrightleftharpoons{\text{phosphorylase}} \underset{\text{(starch or glycogen)}}{(\text{glucose})_n} + n\ (H_3PO_4)$$
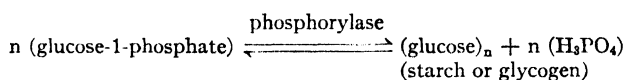
A small amount of preformed polysaccharide is necessary for this reaction, because phosphorylase acts to add new glucose units to existing terminal units in the polysaccharide. Although synthesis of polysaccharides by this method has not been observed in bacteria, it is likely that it does occur.

New glycosidic bonds may also be formed at the expense of existing glycosidic linkages. Such reactions occur with little energy change and are thus reversible. The polymeric degradation of sucrose to form levans and dextrans has already been described. This mode of synthesis is widely distributed in bacteria. Dextran, a polymer of glucose units joined in 1,6-glycosidic linkages, is found in *Leuconostoc mesenteroides* and in some types of pneumococcus. Levans are polymeric 2,6-glycosides of fructose, and levans of close chemical and immunological similarity are present in several species of Bacillus, one of Aerobacter, and two of Streptococcus.[35]

The manner in which the energy for all these syntheses is eventually derived from ATP may be illustrated in a diagram adapted from Avineri-Shapiro and Hestrin[60]:

$$\text{glucose} + \text{ATP} \longrightarrow \text{glucose-6-phosphate} + \text{ADP}$$
$$\Updownarrow$$
$$H_3PO_4 \quad + \quad \text{sucrose} \rightleftharpoons \text{glucose-1-phosphate} + \text{fructose}$$

glucose + levan ⇌—————⫞   ⫞——starch + H_3PO_4

fructose + dextran ⇌————⫞   ⫞——glycogen + H_3PO_4
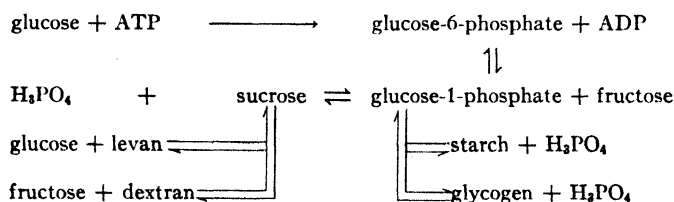
Oxidative Assimilation.[61] In a culture of growing bacteria it is obvious that the constituents of the medium are being converted into bacterial cell material. However, it was not fully realized before the work of Barker and Giesberger that even resting, non-proliferating cells do not oxidize their carbohydrate substrates completely to CO_2 and H_2O but instead assimilate a large portion of

[59] Cori, Swanson, and Cori: Fed. Proc., 1945, 4:234.
[60] Avineri-Shapiro and Hestrin: Biochem. Jour., 1945, 39:167.
[61] Clifton: Advances in Enzymology, 1946, 6:269.

the carbohydrate into cell substance. In the colorless alga, *Prototheca zopfii*, the first organism studied by Barker, 50 to 80 per cent of simple substrates such as acetic acid and ethanol were assimilated into cell substances with the empirical formula of carbohydrate. This phenomenon, called by Barker oxidative assimilation, is of general occurrence in microorganisms and has been observed in several species of bacteria, yeasts and algae. In several experiments, the assimilated material has actually been identified as carbohydrate. Low concentrations of certain cell poisons, sodium azide and 2,4-dinitrophenol in particular, may inhibit the oxidative assimilation of a substrate and allow its complete oxidation to take place, apparently without inhibiting other metabolic processes. It is of interest that both these poisons interfere with t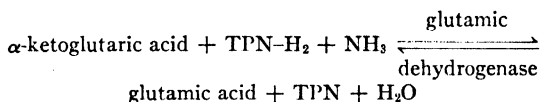he formation of energy-rich phosphate bonds.[62] However, there is as yet no direct evidence that energy-rich phosphate bonds are involved in oxidative assimilation. Clifton has suggested that oxidative assimilation is brought about, not by energetic coupling of synthetic and degradative reactions, but by oxidative degradation of carbohydrate to simple "building blocks" such as acetaldehyde which may be converted into carbohydrate through a series of reactions not requiring energy from an independent system.

Formation of Amino Acids from Carbohydrates. The carbon skeleton of metabolized carbohydrate may be converted into amino acids, purines, pyrimidines, fatty acids, etc. The ability of heterotrophic bacteria to carry out these transformations is well illustrated by the growth of organisms such as *Bact. coli* in a medium containing glucose as the sole organic compound. Although other carbohydrates and carbohydrate derivatives undoubtedly form amino acids, the four-carbon dicarboxylic acids appear to be especially active in amino acid formation. Oxalacetic and α-ketoglutaric acids may be reductively aminated to aspartic and glutamic acids in bacteria (p. 109), and the formation of glutamic acid has been studied in detail.

$$\alpha\text{-ketoglutaric acid} + TPN\text{-}H_2 + NH_3 \underset{\text{dehydrogenase}}{\overset{\text{glutamic}}{\rightleftarrows}}$$
$$\text{glutamic acid} + TPN + H_2O$$

Amino acids may also be formed from carbohydrate in transamination reactions.

$$\text{oxalacetic acid} + \text{glutamic acid} \rightleftarrows \alpha\text{-ketoglutaric}$$
$$\text{acid} + \text{aspartic acid}$$
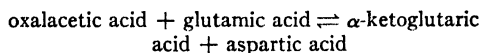
Regulation of Carbohydrate Metabolism. Higher organisms possess complex regulatory mechanisms which determine the pathways and control the rate of carbohydrate metabolism. These mechanisms are largely hormonal in nature. Bacteria possess no such formidable regulatory mechanisms, but even in bacteria the metabolism of carbohydrate is governed by the environment in which the cells are suspended. The temperature, the pH of the medium, the concentration of available substrate, and the presence or absence of oxygen may affect the rate of metabolism and the nature of the end products produced.

A classical example of such a controlling factor is the effect of molecular oxygen upon the metabolism of organisms with both aerobic and anaerobic

[62] For literature citations, see Lardy and Elvehjem: Ann. Rev. Biochem., 1945, *14*:16.

mechanisms for carbohydrate metabolism—that is, the facultative anaerobes. Early in his studies on the fermentative activities of microorganisms, Pasteur observed that the anaerobic fermentative breakdown of carbohydrate is lessened in the presence of oxygen. Many years later, Warburg called this phenomenon the Pasteur effect or Pasteur reaction.[63] The Pasteur effect has been studied extensively by Meyerhof, Warburg, Lipmann and many others, who have found that oxygen inhibits fermentation in almost all facultatively anaerobic cells, whether they are bacteria, yeasts, or the cells of higher plants and animals. The value to a facultative anaerobe of such a regulatory mechanism is obvious. By means of the Pasteur effect, the anaerobic fermentative breakdown of carbohydrate is blocked in the presence of oxygen, and energy is furnished by the far more efficient aerobic oxidation of carbohydrate. However, the actual mechanism of the Pasteur effect is far from obvious and is still the subject of spirited controversy. At present, it is generally believed that anaerobic fermentation is not inhibited by aerobic respiration but by the direct action of molecular oxygen upon some portion of the anaerobic breakdown mechanism.

NITROGEN METABOLISM[64]

The metabolism of nitrogenous compounds by bacteria is less well understood than that of the carbohydrates, and the decomposition of these compounds is somewhat more complex in that the ring structures of the aromatic amino acids make their breakdown a series of special cases. In general, the decomposition of proteins and amino acids is of less quantitative importance in the processes of respiration except in certain cases such as that of the obligate anaerobes. The metabolism of these compounds is of no less importance, however, and the significance of the mechanisms of synthesis, which are intertwined with those of carbohydrate synthesis, is becoming increasingly clear, not only in relation to particular questions such as that of the mechanism of action of the chemotherapeutic drugs, but also in that the microorganisms have certain unique advantages in the investigation of the general biological problems of anabolism.

The Hydrolysis of Proteins. The ability to make use of amino acids is widespread among the bacteria, with the formation of compounds such as hydrogen sulfide, indol, amines and the like which are associated with the decomposition of proteins. The ability to hydrolyze native proteins to their amino acid constituents is, however, one that is possessed by relatively few kinds of bacteria. The limited occurrence of this capability is sufficiently marked that it is customary to speak of bacteria as fermentative or proteolytic in type according to whether carbohydrate oxidation or proteolysis characterizes their biochemical activities.

As compared with knowledge of the proteolytic enzymes of higher organisms, very little is known of the bacterial proteases.[65] In general they appear

[63] See the reviews by Burk: Cold Spring Harbor Symposium on Quantitative Biology, 1939, 7:461; and Lipmann: in *A Symposium on Respiratory Enzymes.* University of Wisconsin Press, Madison. 1942. p. 48.

[64] See the review by Gale: Ann. Rev. Microbiol., 1947, 1:141.

[65] See Haines: Biol. Rev., 1934, 9:235.

˚to be tryptic in nature, *i.e.,* they act in slightly alkaline media, whereas in protozoa and probably in fungi, pepsin-like enzymes occur.) It is probable that a number of proteolytic enzymes are possessed by bacteria analogous to those occurring in higher organisms.(Available evidence indicates, for example, that decomposition may be carried to the polypeptide stage by one or more enzymes, while further dissolution is accomplished by means of another enzyme or group of enzymes. ⌣

The bacterial proteases are, for the most part, extracellular enzymes which are diffused into the medium and are present in sterile filtrates of cultures. Presumably protein molecules are too large to diffuse into the bacterial cell and are partially hydrolyzed outside. Split products probably diffuse into the cell to be further decomposed. Intracellular bacterial proteases have, however, been found.[66]

⌐Of the bacterial enzymes the proteases appear to be the most markedly affected by the medium upon which the organisms producing them are grown.⌐Chief among the factors influencing their production is the nitrogenous material present in the medium; in general, highly active filtrates may be obtained only from cultures in protein-containing media, although the detection of proteolytic activity in cultures grown in some of the so-called "synthetic" media containing no protein material indicates that the presence of substrate is not essential to the formation of the enzymes. The inorganic salts present are also of importance. It has been shown, for example, that the presence of magnesium and calcium salts is necessary for the production of gelatinase, the former stimulating growth but the latter actively stimulating the production of the enzyme. Undoubtedly a series of other environmental factors affect the production of proteolytic enzyme, but present knowledge is extremely fragmentary. ⌣

The ability to liquefy gelatin, a character of considerable value in the biochemical differentiation of bacteria, is often taken as indicative of the proteolytic potentialities of these organisms. Many organisms, however, which liquefy gelatin are unable to break it down further to amino acid units which may be used. *Staphylococcus aureus,* for example, although liquefying gelatin, is unable to hydrolyze the protein to abiuret substances and, presumably, can neither assimilate nor oxidize this substance. The digestion of gelatin by actively proteolytic organisms such as *Proteus vulgaris* is, on the other hand, carried rapidly to the amino acid stage, and evidences of the further decomposition of the amino acids, such as the liberation of ammonia, soon appear.

The resistance of pure native proteins, coagulated proteins and even purified proteose to attack by actively proteolytic bacteria in the absence of other food materials is well established. Such bacteria, inoculated into a mineral salt solution containing a pure protein such as crystalline egg albumin, do not multiply but in time die off. If, however, a small amount of peptone or meat extract is added to such a medium, the bacteria grow well and decompose the pure protein in a normal way. It is probable that extracellular protease inoculated with the bacterial cells is thereby so diluted that no hydrolysis takes place and, in the absence of smaller molecules that can diffuse into the cells
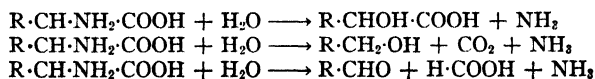
[66] Avery and Cullen: Jour. Exp. Med., 1920, 32:547, 571.

and be broken down by enzymes concentrated there, the bacteria starve to death. Added food material such as peptone may be regarded simply as nutriment which makes possible the initiation of growth rather than as substances essential to the formation of proteolytic enzymes.

The evolution of ammonia which accompanies the bacterial decomposition of protein material is considerably curtailed if a fermentable carbohydrate is included in the protein-containing medium in which the organisms are grown. This phenomenon has been interpreted to indicate that carbohydrate exerts a "protein-sparing" effect on bacterial proteolysis analogous to its protein-sparing effect on mammalian metabolism. Precise work, however, has shown that the analogy is spurious. A diminution in ammonia found might be due to either a decreased production or an increased utilization, since ammonia is the chief source of nitrogen for the great majority of bacteria. The presence of readily available energy in the form of a fermentable sugar stimulates growth with a coincident increase in ammonia assimilated and, in consequence, the ammonia present is diminished.

The Decomposition of Amino Acids. As pointed out above, with very few exceptions, such as the autotrophic organisms, all bacteria are able to utilize amino acids, and in most cases the decomposition of amino acids present in an actively growing bacterial culture proceeds rapidly. The enzymes concerned in the primary utilization of amino acids have been studied at some length; knowledge of them is reviewed by Gale.[67] Possibly these compounds may be used directly in some cases as building stones for bacterial protein; but in most instances some type of decomposition occurs which may liberate energy to the organism, and some of its products, such as ammonia, are the starting points of bacterial synthesis. Amino acids may be broken down in a variety of ways which may be summarized as follows:

(1) Hydrolytic decomposition which may result in the formation of a lower fatty acid and ammonia, of an alcohol, carbon dioxide and ammonia, or of an aldehyde, a lower fatty acid and ammonia by the following reactions

$$R \cdot CH \cdot NH_2 \cdot COOH + H_2O \longrightarrow R \cdot CHOH \cdot COOH + NH_2$$
$$R \cdot CH \cdot NH_2 \cdot COOH + H_2O \longrightarrow R \cdot CH_2 \cdot OH + CO_2 + NH_3$$
$$R \cdot CH \cdot NH_2 \cdot COOH + H_2O \longrightarrow R \cdot CHO + H \cdot COOH + NH_3$$

which are often brought about by aerobic organisms. The decomposition to an alcohol is frequently brought about by bacteria.

(2) Decarboxylation with the formation of the corresponding amine (the so-called ptomaines including pentamethyldiamine or cadaverine from lysine and tetramethylenediamine or putrescine from ornithine or arginine) is brought about by a variety of bacteria.

$$R \cdot CH \cdot NH_2COOH \longrightarrow R \cdot CH_2 \cdot NH_2$$
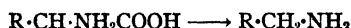
The bacterial amino acid decarboxylases have been studied in detail by Gale.[67] He has shown that six amino acids are commonly decarboxylated, lysine, ornithine, arginine, tyrosine, histidine and glutamic acid, all having in common a free carboxyl group in the one position, a free α-amino group, a free terminal polar group, and the natural *levo* configuration. It has also

[67] Gale: Bact. Rev., 1940, 4:135; Advances in Enzymology, 1946, 6:1.

been reported that aspartic acid is decarboxylated to β-alanine in symbiotic nitrogen fixation, and the decarboxylation of tryptophane has been reported also. It is of interest that pyridoxal phosphate is a coenzyme for the decarboxylases studied by Gale.

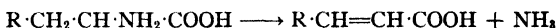(3) Reductive deamination with the formation of saturated fatty acids and ammonia

$$R \cdot CH \cdot NH_2 \cdot COOH + H_2 \longrightarrow R \cdot CH_2 \cdot COOH + NH_3$$

or

$$R \cdot CH \cdot NH_2 \cdot COOH + H_2 \longrightarrow R \cdot CH_3 + NH_3 + CO_2$$
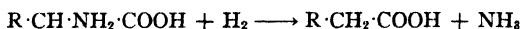
with the formation of the saturated hydrocarbon. These reactions are brought about by anaerobic bacteria which may, for example, decompose glycine to acetic acid and ammonia, or methane, carbon dioxide and ammonia.

(4) Deamination and desaturation at the α-β linkage with the formation of the unsaturated fatty acid and ammonia

$$R \cdot CH_2 \cdot CH \cdot NH_2 \cdot COOH \longrightarrow R \cdot CH = CH \cdot COOH + NH_3$$

is also carried out by anaerobic bacteria.

(5) Oxidative deamination with the formation of ammonia, carbon dioxide and a fatty acid of one less carbon is brought about by aerobic organisms.

$$R \cdot CH \cdot NH_2 \cdot COOH + O_2 \longrightarrow R \cdot COOH + NH_3 + CO_2$$
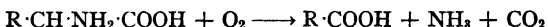
(6) Anaerobic oxidative deamination accompanied by the evolution of hydrogen and the disruption of the amino acid molecule has been described. The products of decomposition of glutamic acid have been determined. The

$$5 \, C_5H_9O_4N + 6 \, H_2O \rightarrow 6 \, CH_3 \cdot COOH + 2 \, CH_3(CH_2)_2 \cdot COOH + 5 \, CO_2 + H_2 + 5 \, NH_3$$
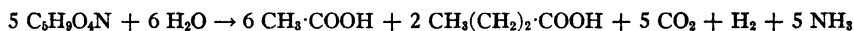
reaction probably proceeds by stages which are yet unknown. This type of decomposition is sometimes known as the "fermentation" of amino acids.

(7) The mutual oxidation and reduction of pairs of amino acids resulting in deamination and decomposition, undoubtedly an important source of energy to some obligate anaerobes and possibly facultative and aerobic organisms as well. Glycine, for example, is reduced to acetic acid and ammonia, and alanine oxidized to acetic acid, carbon dioxide and ammonia

$$CH_3 \cdot CH \cdot NH_2 \cdot COOH + \tfrac{1}{2} O_2 \longrightarrow CH_2 \cdot COOH + NH_3 + CO_2$$
$$CH_2 \cdot NH_2 \cdot COOH + H_2 \longrightarrow CH_3 \cdot COOH + NH_3$$
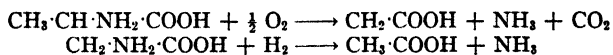
The reactions are, of course, an oxidative and a reductive deamination respectively, but the significance lies in the fact that one amino acid may act as hydrogen acceptor in the oxidation of another. The paired oxidation-reduction of amino acids has been noted earlier in connection with respiration.

The aromatic amino acids are usually attacked first at the side chain, while the ring may or may not be broken. A number of organisms decarboxylate histidine to the physiologically active substance histamine and carry the decomposition no further. The breakdown of tryptophane to indol by some bacteria and not by others is taken advantage of in the biochemical differ-

entiation of many species from one another. Tyrosine may be decomposed to phenol or broken down completely, and a number of organisms break the pyrrolidine ring of proline without difficulty. Owing to differences in ring structure, the decomposition of the aromatic amino acids becomes a series of special cases which cannot be considered further here.

The types of amino acid breakdown outlined above represent a generalization. No single amino acid has as yet been shown to undergo all of the decompositions nor is any single bacterial species capable of bringing about all of these transformations. On the other hand, the bacterial decomposition of an amino acid often involves not a single type of breakdown alone but a series of reactions consisting of several of these types. The nature of the products formed is dependent, to some degree, upon oxygen tension; some compounds such as hydroxy acids may be formed under aerobic conditions but are un stable under anaerobic conditions. The pH of the medium is also of considerable importance; for example, deamination occurs at an alkaline reaction and decarboxylation at an acid reaction. The fact that ammonia is almost always liberated is of considerable significance, for ammonium salts are utilized as a source of nitrogen by the great majority of bacteria.
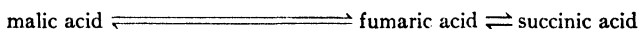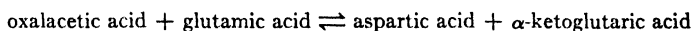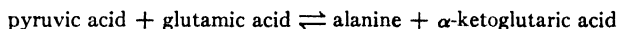
Aerobic and anaerobic protein decompositions are often differentiated by the terms decay and putrefaction. Superficially there would appear to be some justification for regarding the two as separate processes—the justification being largely a matter of the unpleasant odors associated with the reduced products of anaerobic decomposition as contrasted with the lack of odor of the oxidized end products of the aerobic decomposition. Such a distinction has no reality, however, for the processes of decomposition are essentially the same in both cases. Both involve preliminary hydrolysis to amino acids and have further breakdown mechanisms in common. Oxidative deamination, for example, is commonly associated with aerobic organisms, but it also occurs in the oxidation-reduction of pairs of amino acids, a reaction brought about by many of the obligate anaerobes.

Synthesis of Nitrogen Compounds. Not only the autotrophic bacteria but also the most fastidious of the heterotrophes utilize ammonia and ammonium salts as sources of nitrogen. Although, as will appear, amino acids are assimilated directly, they also serve as a source of ammonia through preliminary deamination. The ammonia is taken up and used in the synthesis of amino acids, which are in turn condensed to form proteins.

Until relatively recently little has been known of the methods of synthesis of amino acids. The significance of the anabolic phase of carbohydrate metabolism has been discussed earlier and, as pointed out there, it seems probable that the formation of carbon chains and organic acids, of which keto acids are possibly the most important, constitutes an early stage in amino acid synthesis. Furthermore, the products of glycolysis, particularly pyruvic acid, may also be sources of starting material in amino acid formation. The next stage in synthesis is, of course, the addition of amino groups.

Of the enzyme-catalyzed deaminations, only two, catalyzed by aspartase and glutamic acid dehydrogenase, have been found to be reversible. It would appear, however, that a variety of amino acids could be synthesized from the corresponding keto acids by transamination, the amino group being donated

by a dicarboxylic amino acid. Lichstein *et al.*[68] have found that a number of bacteria can carry out this reaction between glutamic acid and oxalacetic acid to give α-ketoglutaric acid and aspartic acid, between glutamic acid and pyruvic acid to give α-ketoglutaric acid and aspartic acid, and between glutamic acid and pyruvic acid to give α-ketoglutaric acid and alanine. Bacteria such as *Bact. coli* contain all the enzymes necessary to catalyze the following reactions:

pyruvic acid + glutamic acid ⇌ alanine + α-ketoglutaric acid

⇅ CO_2

oxalacetic acid + glutamic acid ⇌ aspartic acid + α-ketoglutaric acid

⇅   ⇅ NH_3

malic acid ⇌ fumaric acid ⇌ succinic acid

The intimate relation between the synthesis of glutamic acid, aspartic acid and alanine with carbohydrate metabolism is apparent. It is possible that transamination systems are a part of a general system of amino acid synthesis, but it is also possible that these reactions are a part of the respiratory mechanism concerned with the metabolism of four and five-carbon acids.

There is also fragmentary evidence of amino acid synthesis from studies on bacteria which require amino acids. There has, for instance, been some study of precursors, *i.e.*, related compounds which can be substituted for the required amino acid, and it has been found, for example, that indol can be substituted for tryptophane to satisfy the growth requirements of typhoid bacilli requiring the amino acid, suggesting that such strains are unable to synthesize the indol ring. More detailed studies with Neurospora mutants have shown that anthranilic acid is a precursor of indol, and that indol is condensed with serine to form tryptophane.[69] Other studies have shown that the synthesis of arginine by Neurospora proceeds through ornithine and citrulline.[70] Still another approach is that of inhibition of amino acid formation by other amino acids or by analogues. The toxic effect of certain amino acid mixtures for the anthrax bacillus reported by Gladstone[71] has been interpreted as an inhibition of synthesis, and similar observations have been made by other workers. The inhibition of methionine formation by analogues has been studied and it has been found that ethionine and methoxinine are inhibitory, and that the inhibitory activity is antagonized by methionine.

It will be clear, however, that as yet there is only fragmentary information regarding the mechanisms of amino acid synthesis. The only general principles indicated, and these are no more than suggested, are those of reversible deamination and transamination.

As yet there is little information regarding the formation of peptide chains and proteins. There is reason to believe, however, that the tendency of glutamic acid to condense into peptides, and the tendency of *p*-aminobenzoic acid, linked

[68] Lichstein and Cohen: Jour. Biol. Chem., 1944, *157*:85; Lichstein, Gunsalus and Umbreit: *ibid.*, 1945, *161*:311.

[69] Tatum and Bonner: Jour. Biol. Chem., 1943, *151*:349; Proc. Nat. Acad. Sci., 1944, *30*:30.

[70] Srb and Horowitz: Jour. Biol. Chem., 1944, *154*:129.

[71] Gladstone: Brit. Jour. Exp. Path., 1939, *20*:189.

through a pterine nucleus to glutamic acid to give the vitamin pteroylglutamic acid (folic acid), are in some way intimately related to peptide and protein synthesis. This assumes considerable significance in view of the antagonism of *p*-aminobenzoic acid for the sulfonamides, and it is possible that these drugs may act through inhibition of synthetic reactions.

The Decomposition of Fats. From the quantitative point of view fats are not an important food source for bacteria. Bacterial lipases are not uncommon, however, and the action of bacteria on fat-containing media has some use as a differential character. Following hydrolysis the glycerol is readily fermented by a wide variety of bacteria, but the ability to decompose the higher fatty acids does not seem to be common and a number of these, such as palmitic, stearic, oleic and other acids, cannot be utilized as a source of carbon by many of the well known bacteria. The oxidative decomposition of fatty acids assumes some importance with regard to the development of rancidity of fat-containing foods.

<center>NITROGEN FIXATION[72]</center>

The ability to use molecular nitrogen as a nutrient is, like the oxidation of ammonia and nitrite, a property of a limited number of bacterial species. Evidence of the biological basis of nitrogen fixation was reported in 1862, and it was later shown that the increase in organic nitrogen content of uncultivated soils could be prevented by sterilization or storing at a low temperature. Following the work of Winogradsky, Beijerinck, Hellriegel and Wilfarth and others it became apparent that atmospheric nitrogen is fixed by

(1) free-living bacteria both

      (a) anaerobic and

      (b) aerobic and by

(2) bacteria living symbiotically with leguminous plants.

The anaerobic organism, *Clostridium pastorianum,* isolated by Winogradsky in 1893, was the first nitrogen-fixing bacterium to be studied in pure culture. When cultivated under anaerobic conditions in a medium containing glucose but no nitrogen, the organism grows well, obtaining its energy through the fermentation of the sugar and its nitrogen from nitrogen gas. The amount of nitrogen fixed is proportional to the amount of glucose fermented—2.4 to 2.9 milligrams of nitrogen fixed per gram of glucose fermented in Winogradsky's experiments. The fixation of nitrogen is inhibited by the presence of ammonium salts in the medium and may be lost through continued cultivation on nitrogen-containing media. *Cl. pastorianum* is a spore-forming rod closely related to the bacteria of the so-called amylobacter group (so-called because many of them stain blue with iodine), organisms which ferment carbohydrate to butyric acid. The chief products of the fermentation of glucose by *Cl. pastorianum* are acetic and butyric acids and carbon dioxide and hydrogen. It has been found that the ability to fix nitrogen may be "restored" to some amylobacter species by a method of "soil passage" which restores this property to strains of *Cl. pastorianum* which have lost it through prolonged cultivation

[72] Biochemical nitrogen fixation is reviewed by Burk and Burris: Ann. Rev. Biochem., 1941, *10*:587; Burris and Wilson: Ann. Rev. Biochem., 1945, *14*:685; Wilson and Burris: Bact. Rev., 1947, *11*:41.

on laboratory media. It is probable that all these organisms constitute a relatively homogeneous group whose natural habitat is soil.

The aerobic nitrogen-fixing bacteria were discovered by Beijerinck in 1901 and named by him *Azotobacter*. Two species were originally suggested: *Azotobacter chroococcum*, a pigmented (deep brown to black), nonmotile organism found predominantly in soil; and *Azotobacter agile*, a motile form producing a soluble green pigment and found in water. Two other species have been found in this country, *Azotobacter vinelandii* and *Azotobacter beijerincki*. Although one or two additional species have been described, these four have, until recently, been the generally recognized members of the genus, with *Az. chroococcum* as typical of the group. This species has a world-wide distribution; the other three are apparently more restricted, although *Az. agilis* may have a wider distribution than formerly thought. All fix nitrogen in alkaline

Fig. 19. *Azotobacter chroococcum*. Three-day culture on nitrogen-free dextrose medium. Nigrosin relief preparation; × 978 (Starkey).

environments, fixation proceeding most rapidly at *p*H 7.0 to 7.5. Starkey[73] has reported the isolation of a species designated as *Az. indicum* from acid soil which fixes nitrogen when grown in an acid (*p*H as low as 3.0) medium. Azotobacter survives for long periods of time, as long as twenty years, and still is able actively to fix nitrogen.

In addition to reaction, a number of other factors govern nitrogen fixation by these organisms. Phosphate, calcium and oxidizable carbon compounds (mannitol or propionate are usually supplied in laboratory media), in addition to certain metals such as iron and molybdenum,[74] are necessary for fixation, and the numbers of these organisms in a given soil may often be increased by the addition of chalk, lime, phosphate or carbohydrate, depending upon the deficiencies of the particular soil.

Like *Cl. pastorianum*, Azotobacter does not fix nitrogen in the presence of adequate amounts of nitrogen compounds in the medium but, unlike the anaerobe, does not lose the ability to fix nitrogen through continued cultiva-

[73] Starkey: Science, 1939, 89:267. See also Starkey and De: Soil Sci., 1939, 47:329.
[74] *Cf.* Burk: Ergebnisse der Enzymforschung, 1934, 3:23.

tion on nitrogen-containing media. Although Azotobacter fixes nitrogen in the free-living state, it is not infrequently found in nature living symbiotically with higher plants. Chlorella, for example, may grow in association with the bacterium, the former assimilating carbon dioxide and the latter molecular nitrogen.

The mechanism by which nitrogen is fixed is obscure. Winogradsky suggested that *Cl. pastorianum* might bring about a direct combination of nitrogen with nascent hydrogen liberated in the fermentation of glucose to form ammonia. It has been generally supposed that the fixation of nitrogen is an endothermic process, but Burk[75] has pointed out that fixation might take place by means of exothermic reactions either in the presence of hydrogen arising from the fermentation of glucose or formate, or as an oxidation in the presence of free oxygen.[76] Wilson and Burris[72] have reviewed more recent evidence
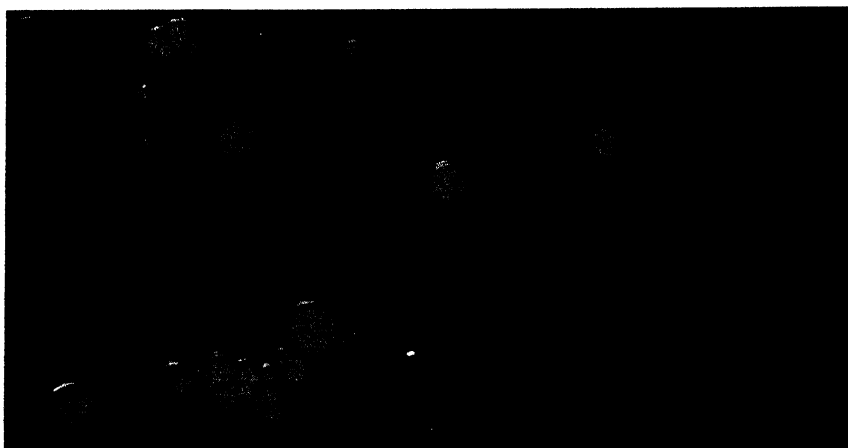

Fig. 20. Colonies of *Rhizobium radicicola* on nutrient agar. Twenty-four hour culture; × 3.

regarding the mechanism of fixation and conclude that ammonia is the most likely intermediate, arising from the reduction of nitrogen by substrate hydrogen. As in other phases of bacterial chemistry, the temptation to assume a free radical chemistry is strong, but there is as yet insufficient evidence to justify such an assumption.

The efficiency of the fixation process is variable depending upon the partial pressure of oxygen. The high respiratory rate of Azotobacter, $QO_2 = 2000$ to 4000, the highest recorded for any living cell, is at a maximum at 10 to 15 per cent oxygen, falling off sharply on either side of this optimum. Maximum nitrogen fixation, however, takes place at 4 to 5 per cent oxygen, and the efficiency of the fixation process increases with decreasing oxygen pressure. Under optimum conditions nitrogen is fixed with an efficiency of approximately 11 per cent of the maximum theoretically attainable, a figure which falls off to about 1 per cent under atmospheric conditions.

[75] Burk: Jour. Gen. Physiol., 1927, *10*:559.

[76] An oxidation to HNO_3. If the concentration of HNO_3 is less than 0.1 molar, $\triangle F$ has a positive value.

The Symbiotic Nitrogen-Fixing Bacteria. The enrichment of a soil by the cultivation on it of leguminous plants such as soy beans, vetch, peas, clover and the like has been known throughout recorded history. Although such plants were early (1838) shown to take up atmospheric nitrogen, the relation between nodule formation and nitrogen fixation was first conclusively demonstrated by Hellriegel and Wilfarth in 1888 and the bacteria present in the nodules were cultivated by Beijerinck in the same year. These organisms, called by him *Bacillus radicicola* and now known under the generic name of Rhizobium, were shown through subsequent work to fix atmospheric nitrogen when living symbiotically in the root nodules. Although the bacteria occur free in the soil, nitrogen is not fixed except in intimate association with the host plant.
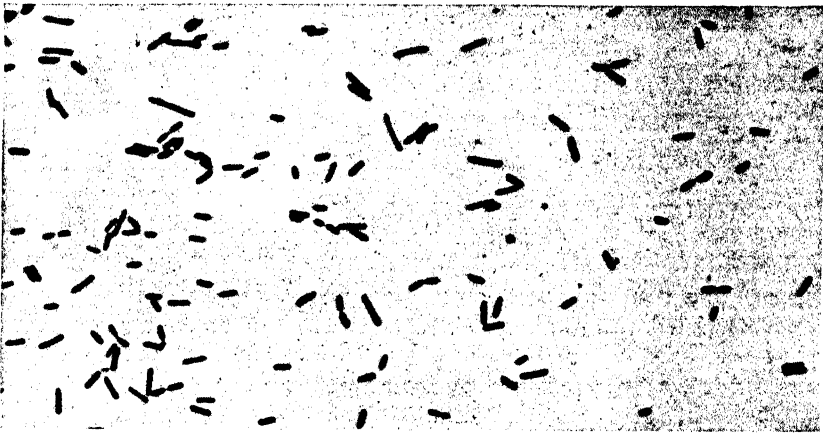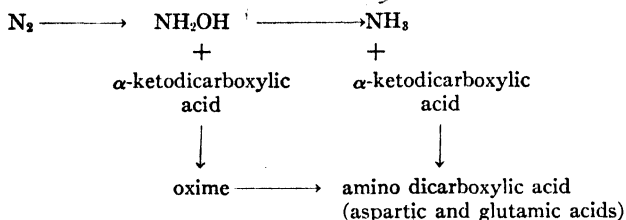

Fig. 21. *Rhizobium leguminosarum.* Smear from a pure culture; fuchsin; × 1050.

The bacteria in pure culture on artificial media are small, motile rods which do not form spores. They show a high degree of pleomorphism in the root nodules, however; small oval forms and branching forms (bacteroides) are found admixed with the rod forms. Some workers regard these bacteria as a single species, *Rhizobium leguminosarum*, which is made up of a variety of strains differing from one another immunologically and in their ability to infect the various species of leguminous plants. These strains fall into two groups, however, one having peritrichous flagella and being somewhat more active biochemically than the other, which has monotrichous flagella. Bergey (1948) recognizes six species of Rhizobium, viz., *Rh. leguminosarum* (legumes), *Rh. phaseoli* (beans), *Rh. trifolii* (clover), *Rh. lupini* (lupine), *Rh. japonicum* (soy beans), *Rh. meliloti* (clover). *Rh. radicicola* is regarded as synonymous with *Rh. leguminosarum.* As a group they appear to be closely related to *Bacterium aerogenes*, Friedländer's bacillus and similar organisms. An organism known as *Bacterium radiobacter* is often found associated with the nodule bacteria but does not fix nitrogen.[77]

In spite of difficulties arising from the fact that the fixation of nitrogen by

[77] For a study of this organism see Hofer: Jour. Bact., 1941, 41:193.

Rhizobium occurs only in association with the host plant, the mechanism of fixation is somewhat better understood than in the case of the free-living nitrogen fixers, chiefly through the work of Virtanen and his colleagues. It has been found that not inconsiderable quantities of nitrogen are excreted by the root nodules during the early stages of growth in the form of glutamic and aspartic acids and β-alanine together with a small quantity of the oxime of oxalacetic acid. It appears that nitrogen is fixed as hydroxylamine, which in turn condenses with oxalacetic acid supplied by the plant. The oxime is then reduced to aspartic acid, which serves as a starting material for the synthesis of other amino acids. The occurrence of glutamic acid has been taken to suggest that hydroxylamine is also reduced to ammonia, but is also compatible with the assumption that nitrogen is fixed as ammonia. The β-alanine found presumably arises through decarboxylation of the aspartic acid. The course of the fixation process may be expressed thus:[78]

$$N_2 \longrightarrow \quad NH_2OH \longrightarrow NH_3$$
$$+ \qquad\qquad\qquad +$$
$$\alpha\text{-ketodicarboxylic} \qquad \alpha\text{-ketodicarboxylic}$$
$$\text{acid} \qquad\qquad\qquad \text{acid}$$
$$\downarrow \qquad\qquad\qquad \downarrow$$
$$\text{oxime} \longrightarrow \quad \text{amino dicarboxylic acid}$$
$$\text{(aspartic and glutamic acids)}$$

Plants other than the leguminosae show nodule formation which, in some cases, is associated with nitrogen fixation. A number of species of alder (Alnus), for instance, have root nodules and there is evidence that nitrogen fixation occurs. Nodule formation is not confined to roots: some plants, the Rubiaceae and Dioscorea, show bacteria-containing nodules on the leaves, and in one instance these have been shown to fix atmospheric nitrogen.

It is open to question whether bacteria other than those described above are able to fix nitrogen. Fixation has been reported for a variety of organisms such as the pneumococcus, but the evidence is inconclusive and fragmentary. It has been shown,[79] however, that the blue-green alga Nostoc is able to fix atmospheric nitrogen.

THE NUTRITIVE REQUIREMENTS OF BACTERIA[80]

It is already apparent that bacteria as a group include a wide variety of physiological types, ranging from the photosynthetic and autotrophic forms to those which metabolize carbohydrates and organic nitrogenous substances by means of complex enzyme systems. The food requirements of these organisms are equally diverse, some being able to utilize inorganic compounds of carbon and nitrogen while others require organic compounds of varying de-

[78] Virtanen: Ann. Rev. Microbiol., 1948, 2:485.
[79] Allison, Hoover and Morris: Bot. Gaz., 1937, 98:433.
[80] The literature to 1936 has been reviewed by Knight: Medical Research Council Special Report Series, No. 210; 1936 to 1938 by Koser and Saunders: Bact. Rev., 1938, 2:99; subsequently in review articles of bacterial metabolism by various authors in Ann. Rev. Biochem., Advances in Enzymology, and Ann. Rev. Microbiol., and most recently by Koser: Ann. Rev. Microbiol., 1948, 2:121.

grees of complexity. The kinds of food materials that must be supplied to an
organism may be considered to fall into three categories:

   (1) compounds that can be oxidized by the organism and thereby serve
        as sources of energy;
   (2) compounds that serve as building stones or as precursors to building
        stones in the synthesis of bacterial protoplasm; and
   (3) substances which, for want of a better name, are designated as growth
        accessory substances or vitamin-like substances and which, in many
        cases, appear to function as components of catalytic systems in the
        economy of the organism.

Although such a classification of food materials is convenient for purposes of
exposition, sharp distinctions are sometimes difficult to make, as will appear.

In general, the utilization of a given substance as a food by an organism
is dependent upon two factors. One, perhaps the more obvious, is the possession
by the organism of enzyme systems which make possible the decomposition or
assimilation of the substance in question. Cellulose, for example, is not a food
for organisms which are unable to decompose it. The second factor is the con-
centration of the substance, which may vary from a lower limit below which
the bacterium is unable to grow to an upper limit beyond which additional
material is simply excess. Very high concentrations may actually be toxic, *viz.*,
the preservation of foods in syrups. Butterfield[81] has shown that between these
two limits a regular relation exists between the amount of bacterial growth and
the concentration of nutrient material; if the logarithms of the maximum
numbers of bacteria are plotted against the logarithms of the concentrations of
food, the points fall on a straight line. The maximum numbers of bacteria are,
in turn, dependent upon the size of the individual organisms, the relation
between size and numbers of individuals being inverse—the smaller the bac-
terium the greater the number of individual cells. When the volume of the
cells is taken into consideration, however, it appears that a given volume of
bacterial substance is formed in a given concentration of nutrient, the larger
numbers of small organisms being but a consequence of wrapping the same
amount of protoplasm in smaller packages.

Oxidizable Substances. The compounds that may serve as sources of
energy to the growing bacterial culture have already been discussed in con-
nection with the mechanisms of oxidation used by these organisms. Suffice
it to say that the autotrophic forms require as food material the inorganic com-
pounds of nitrogen, sulfur and iron that they can oxidize, and that such com-
pounds may be regarded as nutriment in the same way that carbohydrates and
amino acids are regarded as foodstuffs for the heterotrophic forms. The auto-
trophic bacteria, however, exhibit a high degree of specificity with respect to
oxidizable substances which has no counterpart in the metabolism of the
heterotrophic microorganisms. The latter, both as a group and as individual
species, may obtain energy from a great variety of organic compounds of
carbon and nitrogen. As long as such compounds are susceptible to attack by
the enzyme systems of the bacterium, they may serve as food materials.

"Building Stones." The second group of food substances, those that
serve as building stones, may be considered as sources of the chemical ele-

[81] Butterfield: Pub. Health Repts., 1929, 44:2865.

ments which, in appropriate and often highly complex combinations, make up the bacterial protoplasm. Chief among these elements are carbon and nitrogen which the organism may be able to assimilate in a variety of forms. The heterotrophic microorganisms require organic compounds as sources of carbon but do not appear to be particularly fastidious as to the nature of these compounds as long as they may be attacked by the bacterial enzymes. An organism may, for example, assimilate some of the carbon of a fermentable sugar or it may grow normally in the presence of one or more amino acids in mineral salt solutions. The apparent relative unimportance of the nature of the carbon compound suggests that bacteria may break down all manner of carbon compounds to one or more substances which they use as a starting material for the synthesis of cell substance. The intimate relation between glycolysis and the synthesis of carbohydrate and amino acids has been pointed out earlier. The source of nitrogen is either ammonium salts or ammonia liberated by deamination of amino acids supplied in the medium.

The Essential Elements. Elements other than carbon and nitrogen are a part of the bacterial protoplasm and must, of necessity, be supplied in a culture medium. Oxygen is, of course, contained in carbohydrates and amino acids as well as available in the gaseous form to all but the obligate anaerobes. Of the other elements, phosphorus predominates quantitatively because of the high nucleic acid content of the cells. It is generally supplied in the form of phosphate and probably may be assimilated by the organism in the form of other inorganic compounds. A number of other elements, such as iron, magnesium, calcium, etc., have been shown to be necessary to bacterial growth, and certain organisms may have special requirements as in the case of Azotobacter, which requires the presence of elements such as molybdenum and vanadium for nitrogen fixation (which in this case is growth, since the nitrogen is fixed as bacterial protoplasm). It has been found also that iron concentration is of paramount importance in toxin formation by the diphtheria bacillus, the optimum amount being 0.14 micrograms per milliliter.[82] Similar results have been obtained in studies of the formation of tetanus toxin.[83] Iron concentration has also been shown to determine the type of sugar fermentation produced by *Cl. welchii*; with adequate amounts of iron, it is of the acetic acid-butyric acid type with not more than 20 per cent conversion to lactic acid, but with iron deficiency very little volatile acid is formed and 85 per cent of the sugar is converted to lactic acid.[84] Probably a great number of chemical elements go to make up the bacterial cell substance and are, therefore, necessary parts of the food supply, but in the great majority of cases they are required only in the minute traces with which many chemical compounds of the highest purity are contaminated. Obvious technical difficulties have prevented intensive study, but it is quite likely that all of these "biologically rare" elements may be assimilated in the form of inorganic compounds.

The Essential Amino Acids. Bacteria not only require elements such as carbon and nitrogen, but many require them in the form of preformed molec-

[82] Pappenheimer and Johnson: Brit. Jour. Exp. Path., 1936, *17*:335; Pappenheimer: *ibid.*, 1936, *17*:342.

[83] Mueller and Miller: Proc. Soc. Exp. Biol. Med., 1940, *43*:389.

[84] Pappenheimer and Shaskan: Jour. Biol. Chem., 1944, *155*:265.

ular structures. It has been pointed out[85] that the failure of certain bacteria to grow in mineral salt solutions containing fermentable carbohydrate and ammonia might be accounted for on the assumption that such organisms lacked the ability to synthesize certain molecular structures, such as those contained in certain amino acids, and, in consequence, such structures might have to be supplied preformed. The basic assumption is, of course, analogous to the concept of "essential" amino acids of mammalian nutrition. A considerable quantity of evidence indicates that a number of bacterial species require the presence of certain amino acids in order to grow in mineral salt solutions. One strain of the diphtheria bacillus, for example, has been found to require a mixture of tryptophane, cystine, methionine, valine, histidine, glycine and glutamic acid, while another requires leucine, methionine, valine and glutamic acid. *Clostridium botulinum* requires cystine, leucine, lysine, glycine and proline, and another anaerobe, *Clostridium sporogenes*, requires tryptophane, cystine, leucine, methionine, valine, tyrosine, histidine, arginine and phenylalanine. The amino acid requirements of streptococci are similarly complex. The requirements of some other organisms are not so elaborate; some strains of the typhoid bacillus, for instance, will grow in a mineral salt solution plus tryptophane, while others need no amino acids. The significance of observations such as these is not altogether clear at the present time. It is assumed, of course, that such required amino acids are directly assimilated as building stones which the organism is incapable of synthesizing as indicated above; but the demonstration of the oxidation-reduction of pairs of amino acids by obligate anaerobes indicates that these compounds may sometimes function as energy-yielding compounds rather than building stones. Furthermore, analytical evidence[86] suggests that the apparent necessity for the inclusion of amino acids in mineral salt solutions may not be indicative of a lack of synthetic abilities on the part of the microorganisms.

The direct assimilation of amino acids by bacteria has been shown by Gale and his associates,[87] who concluded that an associated fermentation of glucose is necessary for the passage of glutamic acid across the cell membrane, and for the passage of lysine out of the cell. The relation of such assimilation to the amino acid requirements of bacteria is not clear, but it may be noted that here again an intimate relation between glutamic acid and glycolysis is indicated. Indeed, further studies on glutamic acid assimilation have suggested that antibacterial substances such as sulfathiazole, triphenylmethane dyes and surface active compounds interfere either directly or indirectly with its assimilation,[88] an observation perhaps to be related on the one hand to the activity of glutamine as a bacterial vitamin (see below), and on the other to its possible role in the synthesis of amino acids by transamination reactions.

Growth Accessory Substances or Bacterial Vitamins. The third group of food materials, the growth accessory substances, consists of organic compounds which are necessary to growing bacteria only in minute amounts. Presumably these substances cannot be synthesized by the cell, thereby resem-

[85] Burrows: Jour. Inf. Dis., 1933, 52:126.
[86] Burrows: Jour. Inf. Dis., 1939, 64:145.
[87] Gale *et al.*: Jour. Gen. Microbiol., 1947, 1:53, 77, 86.
[88] Gale *et al.*: Jour. Gen. Microbiol., 1947, 1:299, 314, 327.

bling the essential amino acids but differing from them in that the growth
accessory substances apparently function in the cell economy as catalysts
rather than building stones for cell substance proper. The search for such sub-
stances goes back over many years, even antedating the discovery of the
vitamins of mammalian physiology. The great majority of investigations were
based upon a general type of experiment in which a solution which did not
support the growth of the bacterium under consideration was supplemented
by extracts of various plant and animal tissues. Since many of these extracts
permitted growth in the otherwise inadequate solution, it was assumed that
they contained active substances which were variously termed biocatalyzers,
growth hormones and the like. The interpretation of experiments carried to
this point but no further is exceedingly difficult. In very recent years, however,
more precise information regarding these substances has become available.

It is of particular interest that the majority of these substances have proved
to be identical with various components of the vitamin B complex, suggesting
a close relation between the metabolic processes of organisms as diverse as
bacteria on the one hand and mammals and other higher animals on the other.
It is not inappropriate, therefore, to refer to these substances as *bacterial vita-
mins*. Other substances required by bacteria and included in this category are
glutamine, purines such as adenine or uracil, pimelic acid, and others. The
requirements of a number of bacteria have been worked out in some detail[89]
but it should be noted that there may be, and often is, considerable variation
from strain to strain of the same species.

The first bacterium to be studied in detail in this respect was the diphtheria
bacillus which Mueller[90] found to require nicotinic acid and β-alanine, the
latter presumably serving as a precursor of pantothenic acid; some strains
require pimelic acid. *Staphylococcus aureus* was similarly studied by Knight[91]
and found to require nicotinic acid and thiamine and, for anaerobic growth,
pyruvic acid and uracil had to be supplied. It has been found by others that
certain strains require biotin. Similarly, the lactic acid bacteria have been found
to require riboflavin, pantothenic acid, nicotinic acid, pyridoxine and other as
yet unidentified substances, and the hemolytic streptococci must be supplied
with pantothenic acid, pyridoxine, thiamine, nicotinic acid, glutamine and
certain purines.

In other instances precise study has made possible an understanding of
growth requirements already known. Thus, in the case of the hemophilic
organism, the influenza bacillus, it has long been known that two substances
present in fresh blood media are required. One of these, the "X factor," is
heat-stable and associated with hemoglobin, while the other, the "V factor,"
is heat-labile and found in yeast and various vegetable extracts as well as in
blood. It is now known that the X factor may be replaced by hematin and the
V factor by coenzyme I or II.

Other bacteria, however, have much simpler nutritive requirements. The
typhoid bacillus, for example, does not require added growth accessory sub-

---

[89] For detailed reviews see Peterson and Peterson: Bact. Rev., 1945, 9:49; Koser: Ann.
Rev. Microbiol., 1948, 2:121.
[90] Mueller: Bact. Rev., 1940, 4:97.
[91] Knight: Biochem. Jour., 1937, 31:731, 966; *ibid.*, 1938, 32:1241.

stances; most strains of dysentery bacilli and Proteus require only nicotinic acid.

These nutritive requirements are generally believed to be an expression of limitations in the synthetic abilities of the microorganisms, the assumption

B COMPLEX VITAMINS REQUIRED BY VARIOUS SPECIES OF BACTERIA*

Bacterium	Substance Required in Micrograms per ml. of Medium							
	Thia-mine	Ribo-flavin	Panto-thenic Acid	Nico-tinic Acid	Biotin	Pyri-dox-ine	Folic Acid	p-Amino-benzoic Acid
Staphylococcus aureus	0.003			0.20				
Streptococcus hemolyticus C203S	0.001	0.004	1.00	0.10		2.00		
Streptococcus epidemicus X40		0.10	0.50					
Streptococcus hemo-lyticus D-NYS			1.25					
Streptococcus zymogenes						0.50		
Streptococcus lactis R							0.0005	
Pneumococci			1.00					
Shigella dysenteriae				0.10				
Proteus morganii				0.20				
Brucella melitensis	0.02							
Lactobacillus casei		0.04	0.03	0.10	0.0001	0.06	0.00005	
Lactobacillus arabinosus 17-5				0.10	0.00015			0.0002
Clostridium tetani			0.05					
Corynebacterium diphtheriae				1.00				

* Data from various authors; modified from table of Stokes, Gunness and Foster: Jour. Bact., 1944, *47*:293.

being the same as that first postulated for the essential amino acids, *viz.,* the substance in question is an essential metabolite which cannot be synthesized by the microorganism and therefore must be supplied as such. From this point of view there appear to be varying degrees of limitation. For example, the influenza bacillus requires the entire cozymase molecule but most bacteria

require only nicotinic acid or nicotinamide, and given this structure presumably synthesize the remainder of the molecule. The case of thiamine is somewhat similar. The molecule consists of pyrimidine and thiazole (see structural formula), loosely joined. *Staphylococcus aureus* is apparently unable to syn-thesize either ring, but grows in the presence of both pyrimidine and thiazole and is presumably able to form the complete thiamine molecule from its two component parts. Other organisms, such as the influenza bacillus, require the presence of the complete molecule, while still others will grow in the presence of one or the other ring structure, supposedly synthesizing the one not required and joining the two to form the thiamine molecule.

The Function of Bacterial Vitamins. As indicated above, the bacterial vita-mins are in many cases coenzymes or parts of coenzymes that the cell cannot synthesize, and their function in the physiological economy of the cell is that of precursors or component parts of these catalysts. Riboflavin is, of course, the functional prosthetic group of the flavoprotein respiratory enzyme, thiamine is the coenzyme of cocarboxylase, and nicotinic acid or amide a portion of the pyridine nucleotide enzymes. The function of these in the respiratory processes of the bacterial cell has been discussed earlier, and it is self-evident that if these substances cannot be synthesized by the bacterial cell they must be supplied.

The function of some of the other bacterial vitamins is not so clear, in large part, perhaps, because they appear to be associated with catalysis of the much less well known reactions of synthesis. Pyridoxine has been studied at some length and it has been found that a pyridoxal $\rightleftharpoons$ pyridoxamine transforma-tion may be concerned in transamination.[92] It has been shown that both pyridoxine phosphate and pyridoxamine phosphate function as coenzymes with extracts of *Str. fecalis* in the catalysis of transamination between glutamic acid and oxalacetic or pyruvic acids with the formation of aspartic acid and alanine respectively as described earlier. Furthermore, pyridoxal phosphate also activates the enzyme systems involved in the condensation of indol and serine to produce tryptophane referred to elsewhere, and pyridoxine plus car-bon dioxide replaces some of the amino acids required by lactobacilli.[93] Pyridoxal phosphate also functions as a coenzyme in the decarboxylation of amino acids by bacteria. It seems definitely indicated, therefore, that pyridoxine and related compounds function in amino acid decomposition, interconversion and synthesis. Similarly, *p*-aminobenzoic acid has been found to be a part of pteroyl glutamic acid or folic acid[94] and the relation of these substances to peptide formation has been pointed out earlier.

Biotin also appears to play some part in anabolic reactions in that it has been found that the assimilation of ammonia by yeast is dependent on its presence, together with that of glucose and phosphate; it has been suggested[95] that biotin may function as a coenzyme of carbon dioxide transfer by virtue of its ring structure. Glutamine is also associated with carbohydrate metabolism and synthesis in that it stimulates and is used up during glycolysis; McIlwain[96]

[92] Snell: Jour. Biol. Chem., 1944, *154*:313.
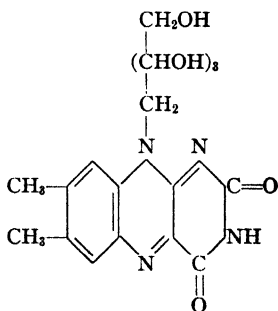[93] Lyman *et al.*: Jour. Biol. Chem., 1946, *162*:173; *ibid.*, 1947, *167*:177.
[94] Angier *et al.*: Science, 1946, *103*:667.
[95] Burk and Winzler: Science, 1943, *97*:57.
[96] McIlwain: Biochem. Jour., 1946, *40*:67.

has expressed the view that glutamine probably acts in the transference of ammonia.

In general, then, while information is quite fragmentary in some areas as yet, it seems clear that the bacterial vitamins are intimately associated with the catalysis of essential metabolic processes, either in respiration or synthesis

riboflavin

COOH.CHNH$_2$.CH$_2$.CH$_2$.CONH$_2$
glutamine

biotin

$$CH_3$$
HOCH$_2$—C—CHOH.CONH.CH$_2$.CH$_2$.COOH
$$CH_3$$
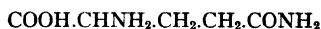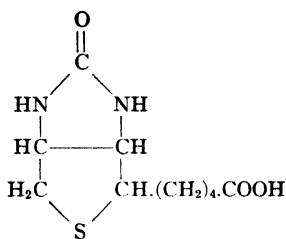
pantothenic acid
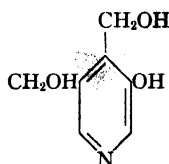
pyridoxine
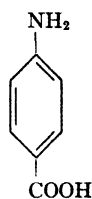
p-aminobenzoic acid
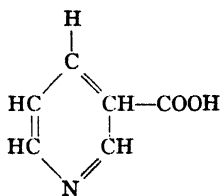
nicotinic acid
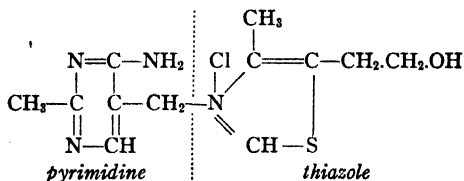
pyrimidine   *thiazole*

thiamine

or a combination of both, since the anabolic and catabolic phases of bacterial metabolism are becoming increasingly difficult to separate.

Carbon Dioxide. The intimate relationship between carbon dioxide tension and bacterial multiplication has long been known. The gonococcus, meningococcus and *Brucella abortus,* for example, require appreciable partial

pressures of the gas for growth, especially in primary isolation, and are usually incubated in vessels containing perhaps 10 per cent carbon dioxide. It is also required by other bacteria, possibly all, for growth though in very much smaller amounts. This may be demonstrated by gassing freshly inoculated cultures with CO_2-free air; if the aeration is carried on vigorously no growth occurs, but if aeration is discontinued it begins. Once growth has been initiated, how- ever, it is not possible to stop it by aeration; presumably carbon dioxide is formed by the metabolizing cells sufficiently rapidly that it cannot be completely washed out in this way.

Studies on the heterotrophic fixation of carbon dioxide described earlier have, however, made clear its essential part in the anabolic reactions, and it is not surprising that it is required as a food substance, so to speak, when it is a part of amino acid synthesis, for example.

THE EFFECTS PRODUCED BY BACTERIAL GROWTH

It has been pointed out in an earlier chapter that bacterial growth may be regarded as a conversion of the constituents of the environment to bacterial protoplasm, a view which is an obvious oversimplification of the mass of highly complex and little understood reactions that constitute synthesis. This con- version is attended by a number of what might be termed "side reactions" which, in many cases, surpass in magnitude the synthesis of bacterial sub- stance. It is to be expected, therefore, that a bacterial culture will contain, in addition to unaltered nutriment, first, materials arising as a result of "side reactions" which are, for the most part, degradations, and, second, bacterial protoplasm together with other substances synthesized by the organisms but not a part of the cell substance proper. The nature of the products so formed depends upon the kind of bacteria present, the kind of nutriments supplied and the conditions under which growth takes place.

The Products of Degradation. Many of these have already been dis- cussed under the heads of carbohydrate and amino acid decomposition. The presence of a fermentable sugar leads to the formation of organic acids and gases such as hydrogen, carbon dioxide and methane. The nature and pro- portions of these end products depend, of course, upon a number of factors, such as the acidity of the culture medium which may favor the formation of one organic acid and tend to suppress that of another; the degree of aeration which markedly influences the degree of oxidation or reduction of the ac- cumulating compounds; the time of incubation, for accumulated compounds may be further decomposed upon continued incubation; and similar factors which exert a strong influence upon what might be considered as the primary determining factor, *i.e.*, the enzymatic equipment of the growing bacterium. Similarly, the hydrolysis of proteins by the proteolytic bacteria and the decom- position of amino acids by these and other organisms to amines, acids, sat- urated hydrocarbons, ammonia, carbon dioxide, hydrogen sulfide, hydrogen, and free nitrogen and oxides of nitrogen is determined by these same factors. A culture medium in which bacteria have grown may, and usually does, con- tain a complex mixture of these various metabolites.

In addition to these, certain other materials, which may also be considered in the category of metabolites, may be present. Chief among them are hydro-

gen peroxide and nitrite, which appear as the end products of coupled reactions in which organic compounds are oxidized and oxygen and nitrate are reduced. Although these substances cannot be reduced by all bacteria, their reduction is in no sense specific to any type of metabolic activity. With the exception of a few organisms such as the Shiga dysentery bacillus and certain streptococci, almost all bacteria produce hydrogen peroxide under favorable circumstances, but, since the majority of aerobic and facultatively anaerobic bacteria possess catalase, peroxide usually does not accumulate in the culture. It is probably not formed through direct coupling of oxygen reduction with substrate oxidation but possibly may arise via some mechanism such as the cytochrome-cytochrome oxidase system. Nitrate is reduced to nitrite by a number of bacteria, and to ammonia by some of the soil organisms, and is often included in simple synthetic media to permit anaerobic growth. The ability to reduce nitrate has assumed some importance as a differential character in the identification of some bacterial species. The reduction of sulfur compounds such as sulfite, thiosulfate, sulfate and the like to hydrogen sulfide is much less common than the reduction of nitrate. Although certain anaerobic bacteria found in muds reduce sulfate, the source of hydrogen sulfide in most bacterial cultures is the sulfhydryl group of cysteine.

The Products of Synthesis. In addition to proteins, lipids and carbohydrate in the usual amounts, many bacteria synthesize relatively large amounts of polysaccharide material which appears morphologically as the capsule surrounding the cell and which may confer an immunological specificity upon the bacterial species (p. 282). Enzymes, are of course, formed, and those that diffuse out of the cells are found in the culture medium. Two other substances have been of particular interest, the pigments produced by the so-called chromogenic bacteria and the soluble toxins elaborated by some of the pathogenic organisms.

Bacterial Pigments. Most bacteria do not form pigments, and masses of the cells, such as colonies on solid media, appear to be white or light gray. Some, however, give rise to colored compounds which may be brilliant in hue. Some pigments occur in solution, while others are found in the form of granules lying outside the cell. Practically all colors of the spectrum are represented: violet, indigo, blue (*Bacterium violaceum, B. janthinus, Pseudomonas pyocyaneus*), green (*Bacterium fluorescens*), yellow (*Staphylococcus aureus, Sarcina lutea*), orange (*Sarcina aurantiaca*), and red (*Bacterium prodigiosum*). The last-named, the so-called "miracle" bacillus, is sometimes the cause of the sudden appearance of blood-red spots on bread, rice, fish and other foods. A single bacterial species may contain more than one pigment; the purple sulfur bacteria, for example, contain lipochrome pigments in addition to bacteriochlorin.

Pigment production is probably one of the most unstable characters of bacteria even though it is often made use of as a differential character in many classification schemes. Some of the staphylococci may show pronounced yellow or golden yellow pigmentation upon isolation from an abscess, but the intensity of the color gradually fades with cultivation on laboratory media and may eventually disappear. Other organisms, such as *Bacterium violaceum*, may also lose chromogenic power through continued cultivation on artificial

media, and cultivation of a chromogenic organism in the presence of dilute antiseptics may result in the complete suppression of pigment formation. Similarly, the incubation of B. prodigiosum at 37° C. results in cultures which entirely lack the brilliant red color characteristic of the organism. As a rule oxygen is indispensable to pigment production and most chromogenic species yield no trace of pigment when grown under anaerobic conditions. *Spirillum rubrum,* however, which grows well in the presence of oxygen, is said to form its red pigment only in oxygen-free media. In the case of some of the chromogens the presence of certain chemical compounds or elements in the medium is essential to, or greatly favors, pigment production. Thus phosphates and sulfates have been found necessary for the production of pyocyanin by *Ps. pyocyaneus,* and sodium tartrate has been shown to favor the production of pigment by *B. prodigiosum.* Carbohydrate media (potato, rice and wheat starch) often lead to a particularly brilliant chromogenesis.

The bacterial pigments are chemically of diverse nature. Many of the red and yellow pigments are insoluble in water, but soluble in alcohol, ether and chloroform, and appear to be lipochromes, a group of fatty pigments widely distributed throughout the plant and animal kingdoms; others, like the fluorescent pigment, are soluble in water, but not in ether or strong alcohol, and may be related to the anthracyanins. Of the bacterial pigments, the structure of pyocyanin is known; it has been found to be an entirely new type of dye and the first instance of a phenazine derivative occurring in nature. The bacteriochlorin found in the purple and green sulfur bacteria appears to be closely related to chlorophyll *a.*

The relation of many of the bacterial pigments to the physiology of the organisms is uncertain. Bacteriochlorin is, of course, responsible for the photosynthetic activity of the purple and green sulfur bacteria. Cytochromes *a, b* and *c* are present in all bacteria except the obligate anaerobes, separately and in various combinations, and presumably function as a part of the respiratory mechanisms operative during aerobic growth. The presence of cytochrome, however, does not impart color to the bacteria. Other pigments such as pyocyanin and phthiocol (a yellow pigment of the tubercle bacillus) are reversibly oxidized and reduced and may function as hydrogen transport systems; there is some evidence that the former may function as an alternative hydrogen transport system to heme systems in azide poisoning.[97] The majority of bacterial pigments, however, fall into the group of lipochromes or carotinoids, and appear to be physiologically inert.

Toxins. The results of the parenteral inoculation of bacteria-free culture filtrates into experimental animals must be interpreted with considerable caution. A great variety of proteins, bacterial and otherwise, yield, on hydrolysis, split products of high molecular weight that are toxic, and the complex amines that may accumulate in the cultures of certain bacteria likewise give rise to marked symptoms, or even death, when injected intravenously. Some bacteria, however, have been shown to form what are called true toxins to distinguish them from the non-specific toxicity of the decomposing material present in the cultures of a variety of bacteria. These toxins diffuse out of the cells and may be found in the culture fluid and are, in consequence, also spoken of as "soluble toxins" or "exotoxins." They are apparently protein in nature, large molecules

[97] Lichstein and Soule: Jour. Bact., 1944, 47:239.

which are synthesized by the organisms capable of forming them, and they have many properties in common with enzymes. The ability to form toxins is one that is possessed by relatively few bacteria, the best known being the diphtheria and tetanus bacilli, the botulinus bacillus and the organism of symptomatic anthrax. These substances are, by a wide margin, the most potent poisons known. There is no explanation for this pronounced toxicity, for the soluble toxins do not differ obviously from other plant and animal proteins. Some of the pathogenic bacteria synthesize other substances termed hemolysins which bring about the dissolution of red blood cells. These substances also appear to be proteins. The cell substance of some bacteria, such as the cholera vibrio, some of the dysentery bacilli and others, is toxic upon injection presumably because of the presence of intracellular or endotoxins. The endotoxins differ in a number of ways from the true toxins, and there is some question as to whether they represent toxic substances synthesized by the bacteria or whether the toxicity of the bacterial cells is a result of the decomposition of bacterial protein to toxic split products. The important subject of bacterial toxins is discussed at length elsewhere (Chapter 8).

The Physical Effects of Bacterial Growth. Both heat and light may be generated by bacterial growth. It has been pointed out earlier that bacteria use only a portion of the energy made available by the oxidation of organic compounds, the remainder being liberated as heat. When circumstances are such that the heat cannot escape as fast as it is generated, the temperature of the bacterial environment rises. The rise may be considerable; the heating of manure piles and damp hay in which bacterial decomposition is proceeding actively may bring them to temperatures as high as 70° to 75° C. Under such conditions the thermophilic bacteria become active and maintain these high temperatures. It has been suggested that spontaneous combustion may result from bacterial activity, but this seems improbable.

The phosphorescence sometimes observed upon decaying fish and meat is due to the growth of light-producing bacteria. The photogenic bacteria are found most commonly, though by no means exclusively, in sea water and upon the bodies of marine animals, and a considerable number of species have been described. Sodium chloride and magnesium chloride favor the growth of the phosphorescent bacteria, and one or the other of these salts is essential to the production of light. Luminescence is apparent only in the presence of oxygen; broth cultures from which dissolved oxygen has been exhausted by the respiratory activity of the organisms may be made to glow by shaking. Bioluminescence in certain crustacea, such as Cypridina, results from the oxidation of a substance, luciferin, by molecular oxygen in the presence of an enzyme, luciferase. Presumably a similar mechanism accounts for the production of light by the luminous bacteria, but neither luciferin nor luciferase has been isolated from these organisms.[98]

BACTERIAL ACTIVITY IN MIXED CULTURES (BACTERIAL ASSOCIATION)

Although the relatively precise knowledge which has made possible the development of bacteriology as a science has been gained through the study of bacteria in pure culture, it must not be forgotten that in nature a pure

[98] Harvey: *Living Light.* Princeton University Press, Princeton. 1940. Also, Ann. Rev. Biochem., 1941, *10*:531.

culture is the exception rather than the rule. Actively decomposing organic matter, for example, contains a great number of bacterial species, and a diseased animal may, and usually does, harbor a variety of microorganisms. The responsibility for a given phenomenon has, in many cases, been fixed upon but one kind of bacterium present in the naturally occurring mixture, the remainder having no apparent significance. The great majority of infectious diseases are phenomena of this type, and it is possible, therefore, to speak of a given bacterium as the causal or etiologic agent of such a disease. Similarly, one microorganism may be shown to be responsible for one kind of fermentation while another takes place as the result of the activity of a quite different bacterium. Modern bacteriology clearly rests upon the generalization that may be made from these facts, the concept of specific microbial etiology. But is this the whole story? It may be asked, first, whether the microorganisms present in a mixed culture are passive with respect to one another or whether one species is affected, either adversely or favorably, by proximity to other actively metabolizing forms; and, second, whether or not a given phenomenon for which no single species can be shown to be responsible may result from the combined activities of a heterogeneous group of bacteria.

Beneficial Associations. With regard to the first of these questions, bacteria have been found to exhibit the various degrees of relationship known to exist among the higher forms of life. Examples of *symbiosis,* a relation in which there is mutual benefit, are rare among the bacteria, but *metabiosis* or *commensalism,* in which one member benefits while the other is unaffected, is commonly observed. Perhaps the most obvious example is the growth of an obligate anaerobe in mixed culture with an aerobic organism. The latter uses up the oxygen in the immediate vicinity, thereby allowing the anaerobe to develop. Sporulating anaerobes are sometimes carried in culture with a non-spore-forming aerobe, for subsequent separation by heat is readily accomplished. In the breakdown of cellulose in the soil, the preliminary hydrolysis by the cellulose-decomposing bacteria yields glucose which may be utilized by a variety of organisms present which are unable to bring about the initial hydrolysis. Similarly, the hydrolysis of protein material by proteolytic bacteria liberates amino acids which may be further decomposed by non-proteolytic forms.

Bacterial Antagonisms.[99] Bacterial antagonism or antibiosis is also of common occurrence. Fermentative and proteolytic types of bacteria generally do not prosper equally well in mixed culture. As pointed out previously, the bacterial proteases are for the most part tryptic in nature and work best in alkaline environments. The acid reaction resulting from the fermentation of carbohydrate is distinctly inhibitory to these organisms, a fact which has been the basis of attempts to replace the proteolytic flora of the large intestine with an aciduric flora consisting of organisms such as *Lactobacillus acidophilus.* The formation of ptomaines or complex amines, whose absorption presumably leads to "autointoxication," by the proteolytic organisms is thereby suppressed or prevented. Numerous other examples of bacterial antagonism have been reported, many of them apparently somewhat more specific than acid inhibition. The well known overgrowth of the diphtheria bacillus by *Staphylococcus*

[99] Waksman: *Microbial Antagonisms and Antibiotic Substances.* Commonwealth Fund, New York. Revised edition, 1947.

aureus is the result of an antagonism as shown by Jennings and Sharp.[100] Other organisms are similarly antagonistic to the diphtheria bacillus; in mixed culture with Friedländer's pneumobacillus, for example, no diphtheria toxin is formed, and mixed infections with these two organisms usually run a mild course. Both *Bacterium coli* and *Bacillus mesentericus* are antagonistic to the diphtheria bacillus, but other organisms, such as the typhoid bacillus, may have no effect. *Bacillus mesentericus*, in turn, is without effect on a variety of other bacteria. Although antagonistic to the diphtheria bacillus, *Staphylococcus aureus* is itself markedly inhibited by the pneumococcus. Many similar examples of antagonism have been observed among the colon-typhoid group of bacteria, such as that between certain strains of coliform bacilli and dysentery bacilli which is due to the elaboration of a substance, designated colicin, by the former.[101] It is not unlikely that such relationships contribute in part to natural resistance to some kinds of infectious disease.

In general, non-spore-forming pathogenic bacteria are closely adapted parasites unable to maintain a saprophytic existence in nature, and persist for only a limited time apart from the host. This failure to survive is due in part to an unfavorable physical environment and in part to an inability to compete successfully with the free-living microorganisms. Furthermore, it has been found that the free-living forms are, in many instances, actively antagonistic to such pathogens as well as occasionally to one another. Thus the typhoid bacillus will survive two to four weeks in sterile water but less than a week in tap water, and dies out even more quickly in raw river or canal water. Similarly, the pathogens disappear relatively rapidly from soil when deposited there by elimination from the infected individual or by earth burial of persons dying of infectious disease; there is, therefore, no hygienic objection to earth burial. The rate of disappearance of such bacteria is in part a function of the temperature, low temperatures tending to preserve them. It may be markedly accelerated by successive inoculations of a plot or sample of soil, a process which increases by selection the relative numbers of those microorganisms which feed upon or specifically destroy the inoculated bacteria.

Because of its rich and varied population the soil has proved a prolific source of antagonistic microorganisms even without the enrichment of repeated inoculation. The method of isolation of antagonists for a particular bacterium is relatively simple. An agar plate is sufficiently heavily inoculated with the bacterium to produce a uniform film of growth and then streaked with soil. After incubation it will be found that the plate is covered with the film of growth of the inoculated bacteria together with colonies of microorganisms from the soil inoculum. Colonies of microorganisms antagonistic to the bacterium will be surrounded by a clear zone in the film of growth in which growth has been prevented. Such colonies may be picked and the microorganism subjected to whatever further study is desired. Microorganisms which are antagonistic to bacteria are sometimes other bacteria, but more often are higher fungi, molds and actinomycetes. (For the antagonism of higher plants to bacteria see p. 328.)

[100] Jennings and Sharp: Nature, Jan. 25, 1947, p. 133.
[101] See Fredericq and Levine: Jour. Bact., 1947, 54:785; Halbert: Jour. Immunol., 1948, 58:153.

The Antibiotic Substances.[102] Investigation of antagonistic micro-organisms has shown that in many instances the antagonistic effect is due to the activity of a substance formed by the antagonist which is toxic for the affected bacterium. These substances have been termed *antibiotic substances* or *antibiotics* by Waksman. Such substances have been known for many years, the first, pyocyanin, having been isolated by extraction of "blue pus" in 1860 before the causative bacterium, *Pseudomonas pyocyaneus,* was discovered. Many others are now known. In general they are more effective on gram-positive bacteria, and differ from one another with respect to their relative toxicity for various species of bacteria. Their chemical properties indicate that they are of diverse nature, including polypeptides, sulfur-containing compounds, lipids, pigments, quinones and organic bases. Some have been prepared in crystalline form and in a few cases the structure is known. These substances have become of greatly increased interest in recent years since it has been found that, while some are highly toxic to man, others are effective chemotherapeutic agents.

Antibiotic Substances from Bacteria. PYOCYANIN AND PYOCYANASE. The best known antibiotic substances of bacterial origin are those produced by *Pseudomonas pyocyaneus* and by aerobic sporulating bacteria of the genus Bacillus. *Ps. pyocyaneus* forms two substances having antibiotic activity, the chloroform-soluble phenazine compound *pyocyanin,* and a substance known as *pyocyanase,* which appears to be a lipid, whose activity is associated with the presence of unsaturated fatty acids in the molecule. A third substance which also shows activity may be isolated by ether extraction; this is a yellow pigment, *hemipyocyanin,* and a derivative of pyocyanin. These substances are all relatively toxic to higher animals.

TYROTHRICIN. An alcohol-soluble, water-insoluble polypeptide having antibiotic activity, which he named tyrothricin, was isolated by Dubos[103] from a gram-positive aerobic sporulating bacterium, *Bacillus brevis.* Further investigation made possible its separation into two components, *gramicidin* and *tyrocidin,* of somewhat different properties though both contain "unnatural" amino acids of the *d* series. Gramicidin is a large cyclopeptide containing relatively large amounts of tryptophane and is effective only on gram-positive bacteria. A very similar substance, *gramicidin S,* is formed by a thermophilic variety of *B. brevis* which differs in that it has considerable activity against gram-negative bacteria and is a cyclopeptide hydrochloride with one free amino group, no free carboxyl and one hydrochloride residue, made up of one residue each of *l*-ornithine, *l*-proline, *l*-valine, *l*-leucine and *d*-phenylanaline. Tyrocidin is active against both gram-positive and gram-negative bacteria *in vitro,* but its activity is almost completely inhibited by serum proteins. These antibiotics are surface-active substances and their antibacterial activity is perhaps attributable to their destructive effect on the cell wall of the bacterium.

BACILLUS SUBTILIS ANTIBIOTICS. A number of antibiotic substances have been isolated from strains of *B. subtilis* which vary somewhat in their properties and antibiotic activity, though all appear to be polypeptide in nature. *Subtilin* is effective chiefly on gram-positive bacteria and certain of the acid-fast

[102] See Benedict and Langlykke: Ann. Rev. Microbiol., 1947, *1*:193; Bailey and Cavallito: *ibid.,* 1948, 2:143.

[103] Dubos: Jour. Exp. Med., 1939, 70:1.

bacilli. It has low toxicity and is effective in the therapy of experimental infection of mice with pneumococci and guinea pigs with anthrax, and may prove to be an effective chemotherapeutic drug. *Bacitracin* is similar to subtilin and effective on gram-positive bacteria. It is somewhat toxic but non-irritating to tissue and has possibilities as a chemotherapeutic agent. *Bacillin* is highly active on both gram-positive and gram-negative bacteria *in vitro* and is only moderately toxic, but, like tyrocidin, its activity is destroyed by blood and it is completely ineffective as a chemotherapeutic agent. *Eumycin* does not affect gram-negative bacteria but has *in vitro* activity against the diphtheria and tubercle bacilli and some of the fungi. Its toxicity is low but its therapeutic efficiency is not known. *Licheniformin* resembles subtilin in its properties and is regarded by some workers as identical with it.

Other antibiotics from bacteria include *colicin* or *colistatin* referred to above, which are formed by coliform bacteria and inhibit gram-negative bacilli such as the dysentery bacilli. Some of the bacterial pigments show antibiotic activity, including *violacein* from *Chromobacter violaceum, phthiocol* formed by the tubercle bacillus, and *iodinin* from *Chromobacter iodinum,* discussed later in connection with competitive inhibition. A substance named *diplococcin* has been obtained from milk streptococci which is active against species of Bacillus, Clostridium, Lactobacillus and Streptococcus and which has considerable chemotherapeutic activity in experimental streptococcus infections. None of these have been fully studied.

Antibiotic Substances from Actinomycetes. A number of antibiotic substances have been isolated from actinomycetes, especially by Waksman and his co-workers. The more important of these may be considered briefly. The first to be isolated were *actinomycin A* and *actinomycin B,* isolated from cultures of *Actinomyces antibioticus.*[104] The two substances, soluble in organic solvents, were both prepared in crystalline form, and the former found to be markedly bacteriostatic and weakly bactericidal, while the latter was chiefly bactericidal. Both are highly toxic to higher animals. Actinomycin A is a red pigment and appears to be a polycyclic nitrogen compound; it is usually referred to simply as actinomycin. The substances *actinomycetin* and *actinomyces lysozyme* are formed by *A. albus* and *A. violaceus* respectively and differ from other antibiotics in that their action is primarily lytic. The former is a thermolabile protein which is precipitated by alcohol, acetone and ammonium sulfate, and the latter is a water-soluble, thermostable substance very similar to egg white lysozyme (avidin) but not identical with it.

STREPTOTHRICIN. This antibiotic was isolated from cultures of *A. lavendulae* by Waksman and his co-workers. It is adsorbed on charcoal and may be eluted with mineral acids, is thermostable and precipitated from aqueous solution by alcohol. It is an organic base and has been prepared as the crystalline reineckate which contains no methoxy, methyl or hydrolyzable acetyl groups, and little is known of its structure. It is more active on gram-negative than on gram-positive bacteria, is bactericidal as well as bacteriostatic, and has considerable fungistatic and fungicidal activity. It is of very low toxicity and has therapeutic promise but has not been tested extensively.

[104] Waksman and Woodruff: Jour. Bact., 1941, 42:231; Waksman and Tischler: Jour. Biol. Chem., 1942, 142:519.

STREPTOMYCIN. Streptomycin was also isolated by Waksman from A. *griseus.* It is very similar to streptothricin but somewhat more active. It has been investigated more fully than any other antibiotic except penicillin, in part because its low toxicity and high activity gave therapeutic promise, and in part because its action on gram-negative bacteria supplements, so to speak, that of penicillin on gram-positive forms. On hydrolysis it yields a basic compound, streptidine, and a disaccharide, streptobiosamine. The latter consists of N-methyl-*l*-glucosamine and a sugar, streptose. The structures of streptidine and N-methyl-*l*-glucosamine have been established, but that of streptose and the points of attachment of the three are as yet uncertain. The accompanying formula is that suggested by Kuehl *et al.*[105] The activity of streptomycin is somewhat less than that of penicillin on a weight basis, is defined as that amount inhibiting

streptidine   N-methyl-l-glucosamine   streptose

* possible point of attachment of streptidine
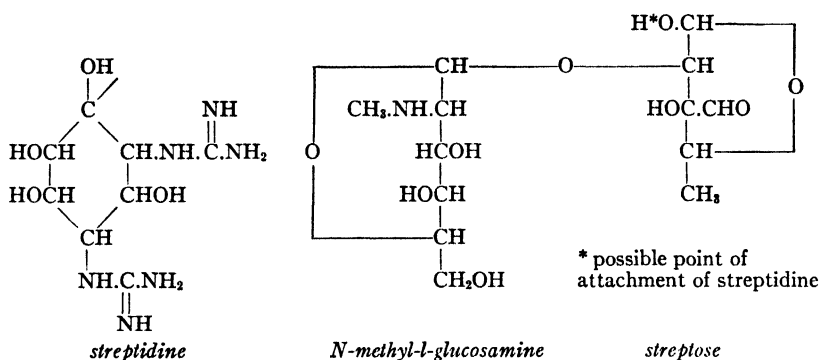
the growth of a test strain of *Bact. coli,* and is assayed in a manner similar to penicillin (see below). There is some tendency to speak of the unit as a microgram, giving the somewhat disconcerting measure of potency of a given preparation as, say, 750 μg. per mg. The mechanism of action of streptomycin is almost completely unknown other than that it is essentially bacteriostatic in therapeutic concentrations. Of the respiratory reactions only the aerobic oxidation of glycerol is inhibited. It is reversibly inactivated by reduction but there is no evidence that it interferes with the functioning of thiol groups in the bacterial cell. It has proven an effective chemotherapeutic agent in certain infections such as tularemia, but must be given in large doses because the bacteria become drug-fast very quickly.[106]

Some other antibiotics are formed by actinomycetes. *Proactinomycin,* formed by *Nocardia gardneri,* is similar to penicillin but somewhat more stable and more toxic. It has been found to have chemotherapeutic activity in experimental streptococcus infection of the mouse. *Nocardine* from *Nocardia coeliaca* has some antibiotic activity against the tubercle bacillus, but only small chemotherapeutic activity. An antibiotic very similar to, and thought by some to be identical with, streptomycin, and designated *streptin* is produced by *Actinomyces sp.*

[105] Kuehl, Flynn, Brink and Folkers: Jour. Amer. Chem. Soc., 1946, 68:2679.
[106] See the review of streptomycin therapy by Keefer *et al.*: Jour. Amer. Med. Assn., 1946, 132:70.

Antibiotic Substances from Molds. PENICILLIN. Of the antibiotic substances produced by molds, *penicillin,* found in cultures of *Penicillium notatum* and produced to a lesser extent by certain other penicillia such as *P. chrysogenum,* is by far the best known. This is largely because it is highly bacteriostatic and almost completely non-toxic for higher animals and has been found to be a highly effective chemotherapeutic agent. It was first found by Fleming in 1929 but its possibilities were not appreciated until the work of Oxford chemists in 1940. Penicillin is readily soluble in water and is extracted from acidified aqueous solution with ether or amyl acetate, and may then be taken out of the organic solvent by shaking with dilute bicarbonate or barium hydroxide solution. It may be purified by chromatographic adsorption and the salts are stable in the dry state.

Very considerable interest has attached to its structure because of its high chemotherapeutic activity. It has been prepared in crystalline form; there is some uncertainty about the structure and the β-lactam form shown here is

$$\begin{array}{c} S \\ \diagup \quad \diagdown \\ NH-CH-CH \qquad C(CH_3)_2 \\ \diagup \quad | \qquad | \qquad | \\ R-C \qquad C-N-CHCO_2Na \\ \diagdown \quad \| \\ O \quad O \end{array}$$
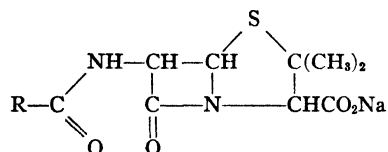
The β-lactam structure of penicillin

generally regarded somewhat more favorably than the alternative incipient azlactone form.[107] Prior to the elucidation of its structure, it was apparent that penicillin is not a single substance in that the product formed by different strains of Penicillium differed. First three fractions, designated F, G, and X in the United States and I, II, and III in Britain, were differentiated, and later a fourth fraction, designated K, was found. The antibiotic produced by *Aspergillus giganteus* is a reduced form of penicillin F, and designated di-hydro F, and the antibiotics produced by *Aspergillus flavus* and originally described as *flavicidin* and *flavicin* were found to differ from penicillin F only in the position of the double bond in the side chain. With the elucidation of the structure of penicillin it became apparent that these penicillins are derivatives of the parent compound shown here with respect to the R group. Thus, penicillin F is Δ^2 pentenyl penicillin, dihydro F is *n*-amyl penicillin, G is benzyl penicillin, X is *p*-hydroxy-benzyl penicillin, and K is *n*-heptyl penicillin. These relationships are summarized in the accompanying table. Of these penicillins, X is the most effective, G and F less so, and K is relatively ineffective, presumably because it is rapidly inactivated in the body.[108]

Penicillin is most active on gram-positive bacteria but some gram-negative forms such as the gonococcus are susceptible to it. It appears to act primarily as a bacteriostatic agent though high concentrations may be bactericidal. In general it is more active than the sulfonamides and less so *in vitro* than the triphenylmethane dyes. Bacteria exposed to penicillin become swollen and fail
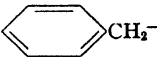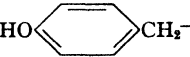
[107] See Clarke, Johnson and Robinson: *The Chemistry of Penicillin.* Princeton University Press. 1948.

[108] See the discussion by Housewright, Berkman and Henry: Jour. Immunol., 1947, 57:343.

to divide, and it is generally believed that some process or processes of cell division are affected. There is, however, no evidence as yet as to the nature of its action with respect to the physiology of the bacterium. It is inactivated by an enzyme, penicillinase, which is produced by a wide variety of microorganisms.[109] It is a highly effective chemotherapeutic agent, especially in staphylococcus and streptococcus infections and certain other diseases such as gonorrhea and syphilis.

Standardization. The antibiotic activity of penicillin is assayed by inhibition of growth of standard strains of staphylococci. Growth may be measured by the turbidity of developing broth cultures, but the most commonly used method is that developed by the Oxford group and makes use of inhibition of

THE NATURALLY OCCURRING PENICILLINS

Name	Synonym	Source	R
penicillin F	penicillin I	*P. notatum* *P. chrysogenum*	$CH_3CH_2CH=CHCH_2^-$
dihydro F penicillin	gigantic acid	*A. giganteus*	$CH_3CH_2CH_2CH_2CH_2^-$
flavicin flavicidin	F type	*A. flavus*	$CH_3CH=CHCH_2CH_2^-$
penicillin G	penicillin II	*P. notatum* *P. chrysogenum*	
penicillin X	penicillin III	*P. notatum* *P. chrysogenum*	
penicillin K	penicillin IV	*P. notatum* *P. chrysogenum*	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2^-$

growth on agar media. It consists of the inoculation of an agar plate so that a uniform film of growth will develop; small cylinders of glass or porcelain are set end down on the inoculated surface. One is filled with standard penicillin solution which contains one *Oxford Unit* per milliliter, and the others with varying dilutions of the unknown. After incubation there is a clear zone of growth inhibition around the cylinders, about 24 millimeters in diameter in the case of that containing the standard solution. The Oxford Unit is, then, defined as "that amount of penicillin which when dissolved in 1 ml. of water gives the same inhibition (*i.e.*, area) as this standard." An International Unit has now been adopted[110] which is defined as the activity of 0.6 microgram of the pure crystalline sodium salt of penicillin II or G, a quantity of which is available as a standard.[111] This unit closely approximates the Oxford Unit.

[109] See the discussion by Woodruff and Foster: Jour. Bact., 1945, 49:7.
[110] League of Nations, Bull. Health Organization, 1945-46, 12:181.
[111] The method of assay used by the Food and Drug Administration is given by Hunter and Randall: Jour. Assn. Offic. Agr. Chem., 1944, 27:430.

NOTATIN. A second antibiotic substance produced by *P. notatum* was described independently by British workers as *notatin* and by American workers as *penatin* and *penicillin B*. It differs from penicillin in that it is insoluble in organic solvents and is active only in the presence of glucose. It appears to be a flavoprotein, producing hydrogen peroxide from glucose, and the peroxide is responsible for its antibiotic activity.

In addition to penicillin and notatin, a wide variety of antibiotic substances have been found to be produced by molds. Some of these are:

ASPERGILLIC ACID. This substance is produced by *Aspergillus flavus* and is active against both gram-positive and gram-negative bacteria. It is an amphoteric substance, soluble in alcohol, ether and acetone but not in petroleum ether, and its empirical formula is $C_{12}H_{20}O_2$. It is very toxic for higher animals.

PENICIDIN. This substance is also active against both gram-positive and gram-negative bacteria and is produced by a number of species of Penicillium. It is extracted from culture fluid by ether at neutrality and precipitated from ether solution by the addition of petroleum ether as a yellow oil. Little is known of its nature.

PENICILLIC ACID. This substance is responsible for the antibiotic activity of some species of Penicillium, notably *P. puberulum* and, in contrast to penicillin, is most effective against gram-negative bacteria. It is water-soluble and has been isolated in colorless crystalline form and found to have the empirical formula $C_8H_{15}O_4$. Two other metabolites of *P. puberulum, puberulic acid* and *puberulonic acid,* are also bacteriostatic. The former has been isolated as a colorless, crystalline dibasic acid with the empirical formula $C_8H_6O_6$, and the latter as bright yellow prisms with the formula $C_8H_4O_6$. Puberulic acid appears to be a quinonoid and puberulic acid the corresponding quinol.

CLAVIFORMIN (CLAVACIN, PATULIN, TERCININ, EXPANSIN). This substance was isolated from *Aspergillus clavatus* and named clavacin, and later from *Penicillium patulum* and called patulin; the two were later found to be identical. The name *tercinin* has been used as a synonym for patulin. It is most active against gram-negative bacteria. It is soluble in ether, chloroform, alcohol and water and has been shown to be anhydro-3-hydroxymethylene-tetrahydro-γ-pyrone-2-carboxylic acid. It shows greater bactericidal activity than most of the antibiotic substances.

FUMIGACIN (HELVOLIC ACID). This substance has been found in cultures of *Aspergillus fumigatus* and is active on gram-positive bacteria. It is soluble in alcohol and sparingly soluble in water and has been isolated in crystalline form.

CITRININ. This substance is formed by *Penicillium citrinum* and acts on both gram-positive and gram-negative bacteria. It is soluble in water and in alcohol and appears to be an organic base.

FUMIGATIN AND SPINULOSIN. These substances were isolated from cultures of *Aspergillus fumigatus* and *Penicillium spinulosum* respectively. Fumigatin has been found to be 3-hydroxy-4-methoxy-2,5-toluquinone, and spinulosin is 6-hydroxy fumigatin. Fumigatin is the more active substance and is effective against such organisms as *Staphylococcus aureus*, the anthrax bacillus and the cholera vibrio.

GLIOTOXIN. This substance is produced by *Gliocladium fibriatum* and some species of Trichoderma and has also been found admixed with fumigatin in cultures of *A. fumigatus*. It is soluble in chloroform and benzol alcohol and sparingly soluble in water. It has been isolated in crystalline form and found to contain both nitrogen and sulfur and possibly an indol nucleus. It is effective on both gram-positive and gram-negative bacteria but unstable except in acid solution.

CHAETOMIN. This antibiotic is formed by *Chaetomium cochliodes* and occurs for the most part in the mycelium. It is extracted from the mycelium with acetone and from the filtrate with ethyl acetate and purified by shaking with bicarbonate, treatment with petroleum ether, and chromatographic adsorption. It is active against gram-positive bacteria but not against gram-negative bacteria.

Bacterial Synergism.[112] Association between bacterial species often affects the organisms in such a way that the ability of the individual species to decompose organic compounds may be modified or extended. This phenomenon, termed bacterial *synergism,* is sometimes qualified into "antagonistic" and "beneficent" synergism, but since the majority of the changes which indicate the combined work of two or more bacterial species cannot be construed as either favorable or unfavorable to the microorganisms, this distinction is often a difficult one to make. Perhaps the simplest examples of synergism are those in which the enzyme system of one organism supplements that of another. A mixed culture of a streptococcus which ferments lactose to acid without gas, and one of the paratyphoid bacilli which does not ferment this sugar, in lactose broth produces an acid and gas fermentation. Similar results have been obtained with mixed cultures of non-sucrose-fermenting *Bacterium coli* and streptococci fermenting sucrose to acid. In such cases the colon-typhoid organisms further oxidize the split products resulting from the decomposition of the disaccharide to gas. Probably similar complementary action is the basis of increased yields of propionic acid in mixed cultures of propionic acid bacteria and lactic acid cocci over that obtained with the propionic acid organism alone. Other decompositions, however, are not so readily explained. Cellulose, for example, is often much more rapidly decomposed by bacteria in mixed culture than it is by component cellulose-decomposing organisms in pure culture.

The course of decomposition may be qualitatively different in mixed culture from that in pure culture. It has been shown, for instance, that the acetone-butyl alcohol fermentation of *Clostridium pectinovorum* is transformed into a lactic acid fermentation by mixed culture with *Bacillus volutans*. A considerable number of pairs of organisms have been shown to produce gas from carbohydrates in mixed culture when neither alone is able to bring about this change,[118] and similarly, mixed cultures of *Clostridium chauvei* and *Bacillus paralactici* form butyl alcohol from glucose although neither alone could produce this change. This ability of mixed cultures of bacteria to bring about

---

[112] See the review by Burrows: *Synergistic Aspects of Bacterial Populations.* Biol. Symp., 1942, 8:89.

[113] Holman and Meekison; Jour. Inf. Dis., 1926, 39:145.

changes of which none of the component species is capable is of interest in connection with the concept of emergent evolution.

One consequence of bacterial association has been of considerable interest in regard to mixed and secondary infections—the alteration of the virulence of a pathogenic bacterium by association with other microorganisms. This alteration may be either a decrease or an increase in the pathogenic powers of the organism. The mild course of mixed infections of the diphtheria bacillus and Friedländer's bacillus has been referred to above, and in this case decrease in virulence is presumably due to inhibition of toxin formation. It is well known that inoculation with soil containing anthrax spores does not always produce the disease even though pure cultures isolated from the same soil are highly virulent. This apparent decrease in virulence is thought by some to have its explanation in the stimulation of the defense mechanisms of the animal by the extraneous bacteria present in the soil. On the other hand, virulence may often be increased by inoculation of a pathogenic organism in mixed culture. The mixture of *Bacterium prodigiosum,* a non-pathogenic form, with the bacillus of malignant edema increases the virulence of the latter so that sublethal doses for experimental animals become fatal. Similarly the virulence of the hemophilic bacteria (Chapter 25) is readily raised by inoculation of the experimental animal with mixed cultures. One of the most interesting examples of the effect of microbial association on virulence is that of swine influenza, in which it has been shown that the disease is produced through the combined action of a bacterium and a filterable virus, neither of which is able to produce the disease by itself. Phenomena such as this suggest that the etiology of some diseases may be a complex matter.

Knowledge of bacterial associations and the mechanisms which give rise to the observed results is in an unsatisfactory state. Experimental evidence is fragmentary, not in the sense of paucity of observation—the literature on the subject is a voluminous one—but because of a certain discontinuity which makes generalization difficult if not impossible. The interpretation of experimental results obtained with pure cultures is often difficult, and when mixed cultures of two or more different organisms are used, the difficulties are greatly multiplied. The loose use of terminology (some workers use symbiosis, metabiosis or commensalism, and synergism synonymously) is no doubt an inevitable consequence of lack of knowledge of the mechanisms involved. In spite of all this, however, the elucidation of many phenomena brought about by bacteria under natural conditions probably lies in the development of knowledge of the association of these organisms with one another.

It may be noted in passing that bacteria may live an associative existence with higher animals and plants. Symbiotic nitrogen fixation and the relation of the cellulose-decomposing bacteria to their herbivorous animal hosts are among the more obvious instances. Infectious disease of man is, of course, an example of antibiosis or antagonism.

Chapter 5

THE EFFECT OF PHYSICAL AND CHEMICAL AGENTS ON BACTERIA

The separation of the environmental factors, that so profoundly affect the life processes of living cells, into two groups, one designated physical and the other chemical, is undoubtedly artificial in many instances. It is often difficult, if not impossible, to differentiate the physical from the chemical; the lethal effect of a germicidal substance, for example, may be a result of a combination of both physical and chemical activity or its mode of action may lie in the borderland of surface and physical chemistry. Nevertheless, such a separation has a certain reality and is useful for purposes of exposition.

Bacteria, like all other living organisms, consist of protoplasm, a delicately balanced, heterogeneous mixture of various substances in colloidal and true solution. The disturbance of this equilibrium through, for instance, the precipitation of constituent protein, is incompatible with the continuation of the complex phenomena of life, and the cell dies. On the other hand, the environment, when favorable, not only does not destroy this equilibrium but makes possible its continuation through growth and multiplication of the organisms. Bacteria, like other organisms, are creatures of their environment; under favorable conditions they multiply rapidly, and under unfavorable conditions either die or remain dormant in a viable state until another opportunity for growth presents itself. In general, they are much more resistant to unfavorable circumstances than are most higher forms of life. A part of this resistance is, of course, a result of the ability of some bacteria to form spores which are relatively highly resistant, but the vegetative cells are considerably more resistant than are the cells of multicellular organisms.

It is not possible to differentiate sharply between these favorable and unfavorable factors. The congruity of a given environmental factor with the protoplasmic equilibrium that is life, is largely a quantitative rather than a qualitative phenomenon. Although high temperatures destroy bacteria, a certain degree of warmth is essential to their growth. Distilled water is toxic to many microorganisms, yet multiplication takes place only in the presence of adequate amounts of moisture. Even the highly active germicidal chemicals often markedly stimulate the growth of bacteria when present in sufficiently low concentrations.

PHYSICAL AGENTS[1]

Temperature Relations. Bacteria as a group will thrive under a relatively wide range of temperature conditions. Some of the more hardy, such as

[1] See the review by Rahn: Bact. Rev., 1945, 9:1.

Bacillus subtilis, will grow throughout the range of 6° to 50° C. Others, such as many of the pathogenic forms, are able to grow over a much narrower range, and some of the more fragile organisms will grow only at body temperature, *i.e.,* 37° C., or very close to this temperature. For all these organisms, however, three temperature limits may be distinguished. There is the *minimum* or lowest temperature at which a given organism will grow, an *optimum* or temperature of most luxuriant growth, and a *maximum,* the highest temperature at which growth can take place. The position of these three points differs greatly among different species of bacteria. In general those organisms whose natural habitat is soil or water have optimum temperatures of 22° to 28° C., while those which, presumably as a result of adaptation to a parasitic mode of existence, cannot survive outside the animal body have an optimum of 37° C.

There are, however, bacteria whose optimum temperatures differ considerably from these. Organisms have been found whose optimum temperatures are from 15° to 20° C., and they have been termed *psychrophiles* or cold-loving organisms. Others having optimum temperatures of 55° to 65° C. may be found in the soil and hot springs and are called *thermophiles.* The great majority of bacteria have optimum temperatures which lie between these two extremes and, in this terminology, are designated as *mesophiles.*

The optimum temperature for given species of bacteria is generally considered to be that temperature at which the organisms grow "best." The question arises as to whether "best" refers to the rate of growth or to the maximum population attainable. It has been found[2] that temperatures optimum for growth in terms of rapidity of cell division are not always the same as those optimum for the attainment of maximum numbers of cells per unit volume. A culture grown at a temperature at which cell multiplication is most rapid will not attain as high a peak in numbers as a culture of the same organism growing more slowly at a lower temperature.[3] Other physiological activities of the cell appear to have optimum temperatures that differ from those which are optimum for multiplication. A given sugar may be fermented to a greater extent, albeit more slowly, when the culture is incubated at a temperature somewhat below that optimum for cell division. Similarly, the anthrax bacillus forms spores most abundantly at 30° to 32° C., while its optimum for vegetative multiplication is 37° C.

The continued growth of bacteria at temperatures somewhat higher than optimum may induce physiological changes of a temporary or permanent character. *Bacterium prodigiosum,* for example, fails to form its characteristic red pigment when incubated at temperatures higher than 30° C., but the change is temporary, for subcultures incubated at lower temperatures form pigment normally regardless of how many transfers have been grown at the higher temperature. The anthrax bacillus, on the other hand, when grown for several transfers at 42° C. loses its ability to form spores and becomes avirulent —a change that appears to be permanent. Incubation or storage of bacterial cultures at temperatures lower than optimum does not result in such qualitative

[2] Graham-Smith: Jour. Hyg., 1920, *19:*131.
[3] See, for example, Spicer: Jour. Bact., 1940, 39:517; Stern and Frazier: *ibid.,* 1941, 42:479, 501.

physiological changes; the metabolic activities of the organisms are slowed down and at temperatures below the minimum for growth the bacteria become dormant.

The mechanisms determining the optimum, minimum and maximum temperatures of bacteria are obscure. In some cases they may be dependent upon other environmental factors. It has been shown, for example, that while many of the thermophilic bacteria are able to grow only at temperatures above 50° C. when in contact with air, they are able under anaerobic conditions to grow at the ordinary incubator temperature (37° C.) or even as low as 34° C. Other studies[4] indicate that the maximum growth temperatures of bacteria bear a definite relationship to the minimum temperature of destruction of respiratory enzymes.
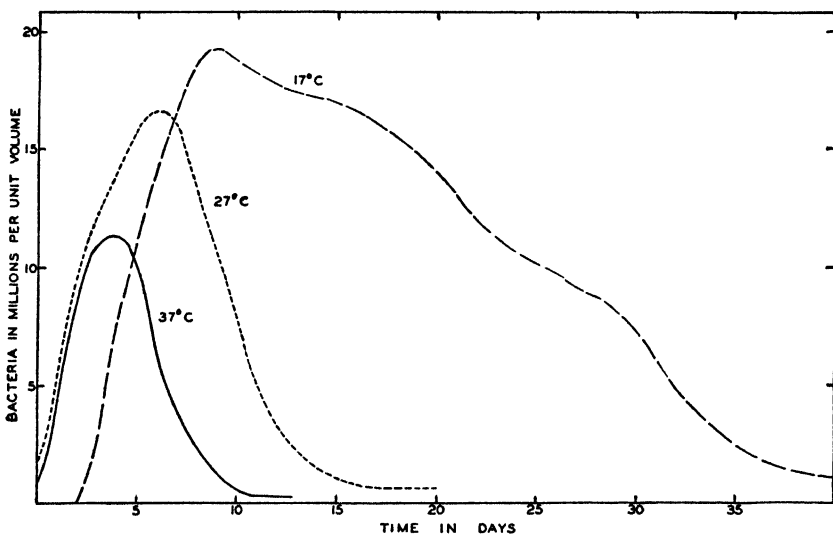
Fig. 22. The effect of different temperatures of incubation on broth cultures of *Staphylococcus aureus*. (After Graham-Smith.)

The rate of bacterial growth and metabolic processes increases, like other chemical reactions, with rise in temperature. The temperature coefficient, *i.e.*, the quantitative effect of temperature changes, is such that these rates are roughly doubled for every ten degree rise in temperature. This effect is observed only over a relatively narrow temperature range, from the minimum to the optimum temperature of the bacterial species under consideration.

The Lethal Effects of Heat. Bacteria are readily killed by heat, and the utilization of heat in one form or another is one of the most convenient means of their destruction. The lethal effects of heat are markedly influenced by the amount of moisture present, and so-called *moist heat* is a much more effective killing agent than dry heat. The resistance of bacteria to moist heat differs somewhat from species to species, the pathogenic forms being in general somewhat less resistant. The resistance of a given species is influenced by

[4] Edwards and Rettger: Jour. Bact., 1937, 34:489.

two important factors, the ability of the organisms to form spores and the previous history of the culture. Spores are always much more resistant than vegetative forms. Some species when in the spore stage can withstand the temperature of boiling water for upward of sixteen hours. The vegetative forms of most bacteria, on the other hand, are killed at 55° to 58° C. by ten minutes' exposure in the presence of moisture. In general the heat tolerance of bacteria appears to be directly related to maximum growth temperatures.[5] The time and temperature of incubation influence, to some extent, the heat resistance of vegetative cells. Actively growing cultures in the logarithmic phase are generally somewhat less resistant than are cells removed from cultures containing maximum numbers of viable organisms. The temperature of incubation appears to affect thermal resistance somewhat, in that cultures grown at and above the optimum temperature are more resistant than cultures grown at sub-optimal temperatures.[6] Previous treatment of both spores and vegetative cells with sublethal doses of ultraviolet light reduces their thermal resistance. However, such changes in resistance are usually of no great magnitude.

Of considerably greater quantitative importance is the pH of the liquid in which the organisms are suspended. Variations from neutrality increase, to a marked degree, the rate at which a given temperature kills bacteria. Acid foods, such as tomatoes, are more readily preserved than foods which have a neutral reaction. Likewise, the addition of sodium carbonate to water in which surgical instruments are boiled increases the efficiency of the heat and at the same time tends to reduce rusting.

The death of an organism from heat is determined not only by the temperature reached but also by the time of exposure. The tubercle bacillus, for example, is killed by thirty minutes' exposure at 58° C., twenty minutes at 59° C. and two minutes at 65° C. One may, therefore, determine the *thermal death* point of a given bacterium by exposure to varied degrees of heat for a constant time; or, similarly, a *thermal death time* may be determined by holding the temperature constant and varying the time of exposure. Both are useful, the latter possibly somewhat more so.

The effect of heat seems to be injurious even when bacteria are not killed, since the cells that have been heated appear to require a longer period of germination. Hershey[7] has shown that exposure to sublethal heat prolongs the latent period preceding cell division without affecting the rate of regeneration of the respiratory function, with no evidence of a period of "recovery" distinct from growth. On the other hand, sublethal heating of bacterial spores often has the effect of accelerating germination.[8]

The application of moist heat to the destruction of bacteria may take several forms. Sterilization by means of steam under pressure is the most efficient of these, because it makes possible temperatures higher than 100° C. in the presence of moisture. At fifteen pounds' pressure, for example, the temperature will be 121.3° C., at twenty pounds, 126.2° C., etc. As a rule, exposure to 120° C. for fifteen minutes suffices for the complete destruction of both vegeta-

[5] *Cf.* Lamanna: Jour. Bact., 1942, 44:29.
[6] *Cf.* Elliker and Frazier: Jour. Bact., 1938, 36:83.
[7] Hershey: Jour. Bact., 1939, 38:563.
[8] *Cf.* Evans and Curran: Jour. Bact., 1943, 46:513.

tive and spore forms of bacteria, although rarely highly resistant spores may be found that are not killed by this exposure. Boiling, *i.e.,* exposure to 100° C., suffices to kill all vegetative forms of bacteria within a few minutes; but sterilization is not effected, for the spores of many of the saprophytic bacilli are not destroyed by boiling for many hours. Since very few of the pathogenic bacteria form spores, boiling contaminated water for a few minutes renders it safe for drinking purposes and the boiling of surgical instruments generally suffices to kill the vegetative cells of pathogenic bacteria, but not the spores of spore-forming pathogens such as those of the tetanus and gaseous gangrene bacilli.

Temperatures as high as 100° C. are not necessary for the destruction of vegetative forms of bacteria, since most of them are killed at 55 to 58° C. if exposed for ten to thirty minutes. In the preparation of vaccines, suspensions of bacteria are usually heated to 60° C. for thirty minutes to one hour (to allow an adequate margin of safety). The tubercle bacillus, *Brucella abortus* and other pathogenic organisms occurring in milk are killed by the process of pasteurization, *i.e.,* heating to 142° to 145° F. for thirty minutes.

Dry heat is much less effective as a germicide than moist heat. Temperatures of 160° to 170° C. must be maintained for two to three hours in order to ensure complete destruction.

The destructive effect of high temperatures on bacteria is apparently associated with the coagulation of constituent protein. The rate of heat coagulation of protein in solution closely parallels the rate of destruction of bacteria by hot water. Furthermore, the effect of the water content of egg albumin on the temperatures necessary for coagulation is similar to the observed relative efficiency of moist and dry heat in the killing of bacteria.[9]

Cold. Bacteria are much less sensitive to low than to high temperatures. As the minimum temperature of a given species is approached and passed, the metabolic activities of the organism become increasingly slower until a state approaching dormancy is reached. Preservation of bacterial cultures in the refrigerator is a common practice, for the rate of death of most bacteria is greatly reduced at low temperatures.[10] Some mortality results from freezing; it is thought to be due to mechanical grinding coincident with the formation of ice crystals. The evidence for this is, however, not altogether indubitable, and attempts to disrupt bacterial cells by alternate freezing and thawing are not particularly successful. These organisms show a remarkable resistance to extreme cold: exposure of organisms like the typhoid and diphtheria bacilli to liquid air (−190° C.) and to liquid hydrogen (−250° C.) does not destroy their vitality. Haines[11] has shown that the temperature at which frozen bacteria are stored significantly influences the death rate. When *Bacterium coli* was kept at −20° C. 25 per cent of the organisms were still alive after 163 days, while at −2° C. death occurred rapidly and only 4 per cent were found viable after eleven days. Death under such circumstances may possibly be a

[9] Egg albumin in aqueous solution is coagulated at 56° C.; with 25 per cent water content at 74–80° C.; with 18 per cent water content at 80–90° C.; with 6 per cent water content at 145° C. Anhydrous egg albumin may be heated to 170° C. without coagulation.

[10] Some species of bacteria, however, are particularly sensitive to cold: gonococci and meningococci, for example, die out much more rapidly in the refrigerator than in the incubator.

[11] Haines: Proc. Roy. Soc., Ser. B, 1938, *124*:451.

result of the denaturation of constituent protein. The viability of pathogenic bacteria in ice from contaminated sources is discussed elsewhere (p. 252).

Drying. The vegetative forms of most bacteria are killed by drying in air, although the different species exhibit pronounced differences in their resistance. The tubercle bacillus is one of the more resistant and the cholera vibrio one of the more sensitive to drying. In general, the capsulated organisms are more resistant than the non-capsulated forms. Spores are quite resistant to drying; the spores of the anthrax bacillus, for example, will germinate after remaining in a dry condition for ten years or more. The resistance of the pathogenic forms causing disease of the upper respiratory tract is of particular interest in connection with air-borne infection (p. 231), for the length of time that a droplet remains infective is a result, primarily, of the resistance of the particular microorganism to drying.

The resistance of a given organism to drying is apparently determined to some extent by the rapidity of the drying process and the temperature at which the organisms are dried and stored. If bacteria are frozen rapidly, as with dry ice and alcohol or one of the glycols, dried from the frozen state (the lyophile process) and the container or ampoule evacuated and sealed, they remain viable with antigenicity and virulence unimpaired for months and years when stored in the refrigerator.[12] It is preferable that they be suspended in a protein-containing solution such as dilute serum before freezing; in the writer's experience broth cultures frequently do not survive. Even bacteria which are sensitive to both cold and drying may be preserved intact by this method.

Light and Other Radiations.[13] The biological effects of electromagnetic radiation of wave lengths greater than 3000 Å are slight and such effects as may be produced by short radio waves and the visible spectrum are largely due to heat. Visible light becomes bactericidal, however, with the incorporation of appropriate dyes in the suspending medium—*photodynamic sensitization*—which promote the absorption of light. The pronounced bactericidal activity of sunlight is due in very large part to its content of ultraviolet light of wave length less than 3000 Å. The wave length 2536 Å shows the highest bactericidal activity and it is perhaps more than coincidence that it is also the wave length at which maximum absorption by nucleic acid compounds occurs. Ultraviolet light is non-ionizing but produces an excitation, *i.e.*, the absorption and dissipation of energy do not result in the ejection of an electron and thus the creation of an ion, but rather raise a constituent electron to a higher stage of energy. In contrast to ionizing radiation, ultraviolet absorption depends on molecular rather than atomic structure, and the excited atoms are distributed at random with no tendency to occur in localized areas. This last probably accounts in part at least for the observation that ultraviolet light is much less effective in killing bacteria than is ionizing radiation.

The ionizing radiations include x-rays, α rays, β rays, γ rays, protons and neutrons. X-radiation is, like ultraviolet light, an electromagnetic radiation but the waves are so short, 0.05 to 10 Å, that there is little or no resemblance in the effects produced. Alpha, β, and γ radiations are emitted by radioactive

[12] Flosdorf and Mudd: Jour. Immunol., 1935, 29:389.
[13] See the general reviews by Lea: Brit. Med. Bull., 1946, 4:24; *Actions of Radiations on Living Cells*. University Press, Cambridge. 1947.

substances. Of these, α rays are nuclei of helium atoms and may also be obtained from the cyclotron; β rays and cathode rays are fast-moving electrons, the latter being artificially accelerated; and γ rays are natural x-rays of short wave length. Protons are hydrogen nuclei moving at high speed; they are not emitted by radioactive substances but are obtainable from the cyclotron. Neutrons, also from the cyclotron, do not ionize directly; fast neutrons project hydrogen nuclei already present in the irradiated material as protons, and when a slow neutron is captured by an atomic nucleus, the nucleus emits an α particle, a proton, an electron, or a γ ray, depending on the element. As ionizing radiation passes through a substance it collides with successive atoms, ejecting electrons from them and leaving a trail of ions behind it. The ejected electrons may have sufficient energy to produce additional ionizations as they collide with other atoms, giving rise to clusters of secondary ionization. When these are small they do not add appreciably to the effect of the primary ionization, and the path of the ionizing radiation takes the form of a column of ionization. Occasionally the ejected electron has sufficient energy to travel an appreciable distance and produce a large number of ionizations in a different path; such secondary electrons are called δ rays and may be of considerable importance, especially in irradiation with α rays. The effect of ionizing radiation, then, is the production of columnar zones of intense ionization in the protoplasm of the irradiated cell.

The bactericidal activity of radiation is due to a direct effect on the protoplasm rather than to the production of toxic substances in the surrounding medium. Such an indirect effect, however, may assume importance under some conditions, *i.e.*, when the nutrient medium in which the organisms are to be grown is irradiated as in the irradiation of bacteria inoculated on the surface of an agar medium. Basically, the bactericidal effect is due to chemical changes in the protoplasm but the precise manner in which it is produced is not completely clear. It seems unlikely that it is a result of the toxicity of irradiated protoplasm, for the magnitude of the chemical changes produced by a lethal dose is not great, and it is generally believed that the lethal effect is due to a direct hit of key substances within the cell, *the target theory*, and their destruction by the zone of intense ionization created. The lethal dose of ionizing radiation is directly related to ion density, or number of ions formed, and for *Bact. coli* has been found to range from 4000 r (r = roentgen, a unit of measurement) for β rays to 24,000 r for α rays. This dose of α rays, for example, corresponds to an average of one α particle traversing each $0.06\mu^2$ of cell substance. The lethal effect of the activation produced by ultraviolet is much less per unit of energy absorbed, and about one hundred times as much energy is dissipated in a bacterium killed by ultraviolet light as in a bacterium killed by x-rays. Wyckoff[14] has found that about 4 million quanta are required to kill a colon bacillus with ultraviolet light.

The lethal dose of either ultraviolet light or ionizing radiation is independent of the intensity of the radiation and of the temperature at which irradiation is carried out, and the survival curves are logarithmic. These suggest that the lethal effect is a single unit action by a quantum of ultraviolet

14 Wyckoff: Jour. Gen. Physiol., 1932, *15*:351.

light or the zone of intense ionization produced by a single ionizing particle, on a key substance in the bacterial cell. In the light of available evidence, the key system affected appears to be the hereditary or nuclear apparatus. Sub-lethal irradiation induces changes in bacteria analogous to radiation-induced mutations in higher organisms such as Drosophila. X-ray induced mutants of bacteria have been studied by a number of workers[15] and, in fact, as yet it is only among x-ray induced mutants of a single parent strain that the gene recombination referred to elsewhere (p. 184) has been found to occur.

Lethal doses of radiation produce no immediately visible change in bacteria, and they continue to be motile, to metabolize, etc. They are, however, unable to reproduce, cell division does not occur and bizarre forms may develop as a result of continuation of metabolic processes other than some key mechanism of division.[16] The manifestation of the lethal effect at the first cell division following irradiation strongly suggests that some mechanism essential to pro-liferation has been destroyed, and what would be termed a lethal mutation in higher organisms has been produced.

In general, bacteria are much more resistant to the effects of irradiation than are other organisms, and the lethal dose may be as high as 100,000 r in some instances as compared to approximately 175 r for the guinea pig and per-haps 800 r for the rat. Simple killing, therefore, does not account for the accelerated healing observed following irradiation of certain infectious proc-esses, and the bacterial toxins show only partial inactivation following mas-sive doses of radiation.

Other Physical Agents. *Sonic and supersonic vibrations* apparently de-stroy vegetative cells upon exposure for sufficient lengths of time. Audible sound waves vibrating at 8900 cycles per second have been found to reduce the numbers of viable organisms in a suspension by 99 per cent in a period of forty to sixty minutes. Ultrasonic waves of 300,000 to 1,000,000 cycles per second have been found to reduce the numbers of viable *Bact. coli* from 70,000,000 to 20,000 per milliliter in fifteen minutes. The destruc-tion is presumably purely mechanical if the effects of rising temperature have been controlled. Many attempts have been made to kill bacteria by pass-ing an *electric current* through their suspensions. If these organisms could be killed in this manner, the sterilization of fluids such as milk would be a simple matter. It appears, however, that an electric current *per se* has no effect on the organisms, although the generation of heat and the liberation of chlorine (arising from the decomposition of chlorides) incidental to the pas-sage of the current may kill the organisms. At *p*H's compatible with life, bacteria are negatively charged with respect to water and respond to an elec-tric current by moving to the anode. The migration of these organisms in an electric field is termed *electrophoresis*. Studies on alterations and reversal of charge have shed some light on the mechanism of the agglutination reaction (p. 301). *Increased pressures* have little or no effect on bacteria unless the

[15] Drea: Amer. Rev. Tuberc., 1938, 38:205; Haberman and Ellsworth: Jour. Bact., 1940, 40:483; Lincoln and Gowen: Genetics, 1942, 27:441; Demerec: Proc. Nat. Acad. Sci., 1946, 32:36; Lederberg: Genetics, 1947, 32:505.

[16] For electron micrograph studies of irradiated cells see Eisenstark and Clark: Science, 1947, 105:553.

pressure is high, 5000 to 6000 atmospheres, and the exposure prolonged. Under such circumstances the organisms may be reduced in numbers. Spores are more resistant; the spores of *B. subtilis*, for example, survive 20,000 atmospheres. On the other hand, animal cells are killed by pressures of 1800 atmospheres. Increased pressures of carbon dioxide show more bactericidal activity, non-spore-forming bacteria being killed after ninety minutes' exposure of 50 atmospheres. Such a germicidal effect may possibly be a result of increased acidity. The sudden release of high carbon dioxide pressures may result in the disruption of the cells of some bacteria; the gram-negative forms are more readily broken up in this manner than are the gram-positive. Sudden release of pressures of other gases does not have this effect.

CHEMICAL AGENTS[17]

The general subject of the effect of chemical agents on bacteria is a broad one and might reasonably be considered as including not only the germicidal chemicals but also the foodstuffs used by these organisms. It is more convenient, however, to consider the latter in terms of the effects of bacteria on their environment in Chapter 4 under the general head of bacterial physiology.

A certain terminology has grown up about this subject matter which requires definition. The synonymous terms *bactericidal* and *germicidal*, which have already been used, are adjectives indicating a bacteria-killing power. The term *bacteriostasis*, or the adjective *bacteriostatic*, denotes a somewhat less drastic effect. A bacteriostatic substance is one which does not kill bacteria but acts in a preservative manner by preventing their growth. An *antiseptic* is a substance which has a preservative action, possibly killing a few of the bacteria exposed to it, but in general acting predominantly in inhibitory fashion. The term *disinfectant* is applied to substances having bactericidal activity and denotes something more vigorous than does antiseptic. These terms are obviously relative, for a substance that is bactericidal in a given concentration may be only inhibitory or antiseptic in lower concentrations. Furthermore, the specificity of a certain compound for some kinds of bacteria may result in its having disinfectant action with regard to one species but only antiseptic action on another.

Water. In general, bacterial cells suspended in distilled water do not survive more than a few hours, although spores will survive for many weeks. Death of the organisms results from a variety of factors one or more of which has been operative in most of the reported experiments. Water from a metal still, for example, often contains sufficient traces of metals to be toxic, and water sterilized in soda or "soft" glass contains alkali dissolved from the glass. Water freshly distilled from hard glass is neutral but upon standing absorbs carbon dioxide from the air and becomes acid. The pH markedly influences the survival of bacteria in water, the death rate increasing on either side of pH 6.0, which appears to be optimum for longevity. Other factors, such as the number of bacteria suspended in a given amount of water, dissolved oxygen and accessibility to oxygen, etc., are likewise known to affect the survival of these organisms. The osmotic pressure of distilled water, which

[17] See Wyss: Ann. Rev. Microbiol., 1948, 2:413.

a priori might be thought of considerable significance in this connection, is not an important factor because bacteria are remarkably resistant to changes in osmotic pressure and are not disrupted or plasmolyzed by suspension in hypotonic or hypertonic solutions.

Acids and Alkalis. Both strong acids and strong alkalis, *i.e.*, those that are highly dissociated, exert a marked bactericidal effect. The lethal activity of the mineral acids is associated with, and proportional to, the degree of their dissociation, but that of the organic acids appears to be an effect of the whole molecule, for the degree of dissociation is, as a rule, not great. The disinfectant action of alkalis such as sodium hydroxide is likewise proportional to the degree of dissociation. The germicidal activity of the hydroxides of the alkaline earths is, however, greater than can be accounted for on the basis of dissociation, for the metallic ion is often toxic in itself. Both acids and alkalis in too low a concentration to kill bacteria rapidly often enhance the activity of other disinfecting agents. For example, the germicidal activity of many salts is greater in the presence of acid or alkali and, as noted above, bacteria are killed much more rapidly by heat in the presence of dilute acid or alkali than at neutrality.

The relation of bacterial growth to the acidity or alkalinity of culture media has been discussed elsewhere. Concentrations of hydrogen or hydroxyl ions compatible with growth are very low, of the order of 10^{-4} to 10^{-9} mols of hydrogen ions per liter. Almost all bacteria will grow at pH 7.0 (1×10^{-7} mols hydrogen ions per liter) but grow best at an optimum which varies from species to species. The minimum and maximum limits between which growth takes place likewise vary widely with species. Certain organisms such as the lactobacilli and *Streptococcus lactis* are termed *aciduric* organisms because they are able to grow profusely at pH 4.0 or less. Perhaps the most acid-resistant organism known is *Thiobacillus thiooxidans*. This organism accumulates sulfate in its cultures as an end product of the oxidation of sulfur and continues to grow even in the presence of N/10 sulfuric acid.

Salts. Salts have, in general, two effects on bacteria. In very low concentrations they markedly stimulate growth and at higher concentrations they become toxic. The particular concentrations at which these effects are apparent are dependent upon the degree of dissociation of the salt, the nature of the anion and the valency and molecular weight of the metallic ion. In general, the bivalent cations are more toxic than the monovalent cations and the salts of the heavier metals are more toxic than those of the lighter metals. There is, however, no precise quantitative relation in either case.

The most active of the heavy metals are mercury, silver and copper. Mercuric chloride is highly active in 0.1 per cent aqueous solution, killing vegetative cells within a short time, and the silver salts, such as silver nitrate, although somewhat less active, are still highly efficient germicides. Copper salts are still less active but are highly efficient in the destruction of algae and other chlorophyll-containing organisms. Dissociation of these salts is intimately related to their disinfectant properties. A solution of mercuric chloride in absolute alcohol has substantially no disinfecting power, but if water be added the germicidal power of the solution increases proportionately to the amount of water added. Because of the importance of ionization, a compari-

son of the bactericidal power of the various metallic salts on the basis of percentage solution is misleading; equimolecular solutions must be used and the ionization constants taken into consideration. The bactericidal activity of the heavy metal salts is a result of the affinity of the cations for protein material; when the constituent protein of a bacterial cell is precipitated as an insoluble proteinate, the cell dies. Other factors appear to be involved also, however. Guest and Salle[18] have observed that inorganic metallic salts which are only slightly bactericidal individually may become markedly active when mixed to produce an oxidation-reduction system.

The *oligodynamic action of metals* is possibly a result of the solution of the metal to form salts. The destruction of bacteria in contact with or in proximity to a piece of metal is the basis of some methods of disinfection of water. Water may be sterilized by allowing it to seep through a layer of silver-coated sand, and water containing colloidal silver in amounts sufficiently small to defy chemical detection, by calculation about 40 gamma per milliliter, is markedly bactericidal. Such colloidal solutions are prepared by sputtering silver electrodes in water—the so-called catadyn process.[19]

The other cations are less toxic than mercury and silver, but even sodium and potassium are toxic to bacteria in sufficiently high concentrations (*ca.* 2 molar). It is of some interest that the arrangement of cations in an order of their toxicity—mercury and silver at one end and sodium and potassium at the other, with others falling in order in between—corresponds closely to the Hofmeister series and the lyotropic series of Freundlich, in which cations are arranged in the order of their effects on physical properties of proteins, such as coagulation, solubility, viscosity, etc. The toxicity of cations as manifested in solutions containing a single salt may often be neutralized by the presence, in the proper proportion, of another cation. This phenomenon, known as the *antagonistic effect of salts*, has led to the concept and preparation of so-called balanced solutions such as Ringer's solution, Locke's solution and others. Salts not only modify or enhance the toxic qualities of other salts but also exert similar effects on disinfectant compounds of widely different constitution. Sodium chloride, for example, markedly enhances the germicidal qualities of phenol for anthrax spores when present in sufficiently high concentrations.

The part which anions play in the growth and destruction of bacteria is less well known. Some, particularly those containing sulfur, carbon, nitrogen or oxygen, may serve as sources of these elements and of energy, but in appropriate concentrations many are toxic for bacteria.

Oxidizing Agents. Other salts, such as potassium permanganate and the sodium and calcium salts of hypochlorous acid ($HOCl$), show marked bactericidal activity owing to their properties as oxidizing agents. Mol for mol, hypochlorous acid is one of the most powerful germicides known, and its calcium salt (commonly known as bleaching powder) has a wide use in the treatment of private and small municipal water supplies. Hypochlorous acid reacts with organic compounds containing an amide group with the formation of compounds known as chloramines. These compounds show strong disinfectant properties which are apparently associated with the presence of the

18 Guest and Salle: Proc. Soc. Exp. Biol. Med., 1942, *51*:272.
19 For a discussion see Hoffmann: Arch. f. Hyg. u. Bakt., 1938, *120*:147.

= NCl group. Two of these, chloramine-T and dichloramine-T, were used with considerable success in the disinfection of deep wounds in the last war. Similar compounds such as the chlorates and perchlorates likewise are bactericidal. The high bactericidal activity of chlorine dioxide has been of considerable interest in connection with its use in the treatment of water supplies, since it is relatively unaffected by alkalinity and has other advantanges over hypochlorites and chlorine for this purpose (p. 259). The halogens, chlorine, bromine and iodine, are also potent germicides but, in contrast to the heavy metals, in inverse order to their atomic weights. Liquid chlorine is widely used in the treatment of water supplies and iodine in the form of its tincture is an efficient skin disinfectant. Bromine has been used occasionally as a disinfectant for swimming pool water. Fluorine compounds are seldom used as disinfectants, although sodium fluoride is toxic to some bacteria, presumably owing to its interference with their oxidative mechanisms. Both hydrogen peroxide and ozone are bactericidal, but the former is rapidly decomposed by tissue catalase and has little penetrating power when applied to wounds and abrasions.

Organic Compounds. More or less successful attempts have been made to utilize the germicidal activity of mercury and silver through the preparation of organic compounds which, while having disinfectant properties, are not markedly toxic to body tissue. These include metaphen (4-nitro-5-hydroxy-mercuriorthocresol), merthiolate (sodium ethylmercurithiosalicylate), mercurophen (sodium oxymercuriorthonitrophenolate), mercurochrome (dibrom-oxymercurifluorescein) and a series of silver-protein compounds such as argyrol, protargol, argonin and the like. The organic compounds of mercury are generally used as skin disinfectants and, in dilute solution, as preservatives. The silver compounds find wide use in the disinfection of mucous membranes, as in the treatment of gonorrhea and eye infections. They are, however, quite ineffective against spores of the obligate anaerobes, Welch's bacillus of gaseous gangrene and the tetanus bacillus.

Aside from these metal-organic compounds, the most efficient organic disinfectants are the coal tar products, phenol, or carbolic acid, and the monomethyl phenols, or cresols. Phenol is commonly used in 5 per cent aqueous solution and at this concentration destroys vegetative cells rapidly and spores somewhat more slowly. The cresols, ortho, meta and para, are not soluble to this extent but in 2.5 per cent emulsions are about three times as bactericidal as 5 per cent phenol. Crude tricresol, a distillate containing various impurities as well as all three cresols, is a more potent disinfectant than any of the cresols alone or a mixture of the three in pure form. Difficulties arising from the low solubility of tricresol may be eliminated by saponification and the end product, lysol, is likewise strongly bactericidal. The germicidal activity of the phenol derivatives may be enhanced by halogen substitution, and if the methyl group of the halogen compound is replaced with aromatic or higher aliphatic groups, toxicity for bacteria is still further increased. Resorcinol, hydroxy phenol, is mildly bactericidal and one of its derivatives, hexyl resorcinol, has gained an undeserved reputation as a general disinfectant.[20]

[20] See the review of the relation of structure of the phenols and their bactericidal properties by Suter: Chem. Rev., 1941, 28:269.

Soaps, the sodium and potassium salts of higher fatty acids, are mildly germicidal in themselves, but probably what disinfectant action they possess may be accounted for on the basis of mechanical removal of microorganisms by emulsification of the lipoidal secretions of the skin in which many bacteria become embedded. The surgical soaps, containing a disinfectant, are not as effective germicides as might be supposed, although somewhat more bactericidal than ordinary soaps.[21] The slight bactericidal action of fatty acids is apparently attributable to the hydrogen ion. During recent years a group of substances similar to soaps, the sodium alkyl sulfates (such as Drene and others), have come into widespread use. These, like soaps, are anionic detergents. Some of these have been found to inhibit the growth of some bacteria in relatively high concentrations (0.1 per cent), but the activity is markedly selective, gram-positive bacteria being inhibited but gram-negative bacteria not affected.

Reduced surface tension in itself has little detrimental effect on bacteria, although it might be supposed that a germicidal substance which markedly reduced the surface tension of water would be more effective as a result of concentration at the bacterium-water interface. In the case of surface-active germicides it has been observed that wetting action and bactericidal proper-

R—COONa

soap

$$R—O\underset{Na—O}{\overset{}{>}}SO_2$$

alkyl sulfate

ties are closely correlated. Whatever the mechanism, it has been found that wetting agents apparently increase the bactericidal action of undissociated phenols.[22] In contrast with the soaps and alkyl sulfates, the salts of quaternary ammonium bases are cationic detergents. These have been of interest in recent years and appear promising as germicides, especially those having 12 to 16 carbon alkyl chains. Very many, perhaps a thousand, of these compounds have been synthesized and are marketed under various trade names such as Phemeral, Zephiran, Roccal and the like. These are definite mixtures of related compounds that differ in the number of carbon atoms in the alkyl chain. They are usually most effective on non-spore-forming gram-positive bacteria such as staphylococci and streptococci, and less so on gram-negative forms such as the enteric bacilli. The high degree of bactericidal activity which they show in the phenol coefficient type of test is, however, largely spurious.[23]

Ethyl alcohol and ethyl ether, often used as skin disinfectants, are not good germicides. Their effectiveness probably lies in the solution of the lipoidal secretions of the skin and consequent mechanical removal of microorganisms. Absolute alcohol has little or no germicidal activity. The bactericidal activity of alcohol-water solutions increases with the addition of water but 50 per cent alcohol and less has little activity; 70 per cent is the concentration usually used for skin disinfection. Absolute propyl and isopropyl

[21] See Morton: Jour. Amer. Med. Assn., 1944, *124:*1195.

[22] See the review of surface-active agents by Glassman: Bact. Rev., 1948, *12:*105.

[23] For a discussion of these compounds see Rahn and van Eseltine: Ann. Rev. Microbiol., 1947, *1:*173.

alcohols are likewise ineffective but show activity in aqueous solution, while absolute methyl alcohol is said to be bactericidal.

Dyes. The dyes are widely used in bacteriology both for staining purposes and as indicators. In addition, many of them show a marked bacteriostatic and bactericidal activity which is often specific in that it is manifested against one organism and not another. The incorporation of an appropriate dye in a medium will render it selective, *i.e.*, it will favor the growth of some species of bacteria and inhibit that of others. In general, this specificity is correlated with the gram reaction; the gram-negative organisms are, for the most part, much less sensitive to dyes than are the gram-positive species. The activity of these compounds is affected by *p*H, the toxicity of the acid dyes increasing with acidity and that of the basic dyes increasing with alkalinity.

Many of the dyes such as the thiazins, oxazins and azo dyes are not particularly toxic for bacteria, dilutions of 1:1000 or less being required to inhibit growth. A number of the triphenyl methane dyes are, on the other hand, inhibitory in high dilutions. Malachite green, for example, inhibits the growth of *B. subtilis* in dilutions of 1:4,000,000 and staphylococci in 1:1,000,000, while higher concentrations, *i.e.*, 1:30,000 to 1:40,000, are required to inhibit the colon and typhoid bacilli. Victoria green, a dichlor derivative of malachite green, is bacteriostatic to about the same degree. Brilliant green is active in even higher dilutions, inhibiting *B. subtilis* in a dilution of 1:15,000,000 and staphylococci in 1:4,000,000 and the typhoid and colon bacilli in 1:500,000.

The bacteriostatic properties of the triamino triphenyl methane dyes, the so-called rosanilins, are apparently associated with the substitution of alkyl groups in the amido side chains. Basic fuchsin, a mixture of the unsubstituted simple dyes rosanilin and pararosanilin, is relatively weakly bacteriostatic, dilutions of 1:500,000 being required to inhibit the growth of *B. subtilis*. Acid fuchsin, a mixture of various sulfonated derivatives of basic fuchsin, is likewise only weakly inhibitory and was formerly widely used in media as an acid indicator under the name of Andrade's indicator. On the other hand, methyl violet,[24] a mixture of tetra-, penta- and hexamethyl paraosanilin, is markedly bacteriostatic and completely inhibits the growth of bacteria such as staphylococci, diphtheria bacilli and others in dilutions of 1:1,000,000 to 1:5,000,000. Approximately 150 times as much dye is necessary to suppress growth of the less sensitive gram-negative bacteria such as the colon and typhoid bacilli. There appears to be a correlation of bactericidal activity and basicity of these compounds. It may be noted that there has been a renewed interest in the dyes as chemotherapeutic agents in recent years.[25]

The acridine dyes, acriflavine and trypaflavine, have been of particular interest because of their therapeutic significance. The former is actively bacteriostatic in dilutions as high as 1:3,000,000 and the latter inhibitive to

---

[24] Gentian violet is a more or less impure mixture of methyl violet and dextrin. Crystal violet, hexamethyl pararosanilin, is one of the constituents of methyl violet.

[25] *Cf.* McIlwain: Biochem. Jour., 1941, 35:1311; Rubbo, Albert and Maxwell: Brit. Jour. Exp. Path., 1942, 23:69; Russell and Falconer: Lancet, 1943, *ii*:580; Browning: Brit. Med. Jour., 1943, *i*:263. Albert, Rubbo, Goldacre, Davey and Starr: Brit. Jour. Exp. Path., 1945, 26:160.

staphylococci and similar organisms in 1 : 2,000,000 and to the relatively fragile gonococcus in dilutions of 1 : 10,000,000 to 1 : 50,000,000. The gonococcus is killed by exposure to the dye in dilutions of 1 : 80,000 to 1 : 400,000 within two or three minutes.

Gaseous Disinfectants. The use of bactericidal gases for the disinfection of rooms, dwellings and the like (fumigation or terminal disinfection) has declined markedly in recent years with no coincident increase in the prevalence of infectious disease. The commonly used gases, formaldehyde and sulfur dioxide (generated by burning flowers of sulfur), are probably not bactericidal as gases but in aqueous solution and are effective, therefore, only in the presence of adequate amounts of moisture (a relative humidity of 60 per cent or higher). Sulfur dioxide in aqueous solution (sulfurous acid) probably owes its germicidal qualities to its acidic nature. Formaldehyde, usually sold under the trade name of formalin (a 33–40 per cent solution of the gas in water), has greater penetrating power and is a more effective germicide than sulfur dioxide. Other gases such as hydrogen cyanide have little or no effect on bacteria. Although the value of terminal disinfection is open to serious question, that of disinfestation is well established and the gases, hydrocyanic acid in particular, are widely used for the destruction of rats aboard ship, etc.

Aerosols. The use of aerosols, bactericidal compounds finely dispersed in the air, for the destruction of air-borne pathogenic bacteria has been developed in recent years, especially by Robertson and his co-workers.[26] These investigators first used propylene glycol as a vehicle for bactericidal compounds but control experiments indicated that the glycol alone (volatilized by heat) was equally effective. Triethylene glycol is the glycol now most commonly used. The glycol is relatively non-toxic and the vapor may be breathed with impunity, but bactericidal concentrations of 50 per cent or higher are attained when droplets come in contact with suspended microorganisms. The rate of destruction of air-borne bacteria is in agreement with the assumption that the glycol molecules in the vapor state condense on the bacteria-containing droplets, and efficacy is associated with a low vapor pressure, high hygroscopicity and, of course, toxicity for the bacterial cell substance.[27] Other workers have used dispersed hypochlorite and ultraviolet irradiation to kill air-borne bacteria but these methods are not too promising. These results are of considerably more than ordinary significance in that they make possible the control of air-borne infection (p. 231); the respiratory diseases have hitherto spread in the human population without hindrance.[28]

The Specificity of Disinfectants. The marked specificity of the bacteriostatic and bactericidal activity of the dyes has been referred to above. The property of differential toxicity is not confined to these compounds alone, however, but is exhibited to some degree by many of the bactericidal chemicals. The hypochlorites, for example, while powerful germicides for most bacteria, have little effect on the tubercle bacillus. In general, the salts of the heavy metals are least specific in their action and dyes the most, with other

[26] Robertson *et al.*: Jour. Exp. Med., 1942, 75:593; *ibid.*, 1943, 78:387; Science, 1943, 97:142.

[27] Puck: Jour. Exp. Med., 1947, 85:729, 741.

[28] See, for instance, the review in Bull. U. S. Army Med. Dept., 1946, 5:538.

compounds lying between these two extremes. Certain slow oxidizing agents such as potassium dichromate exert a selective bacteriostatic effect on gram-negative bacteria, and iodine is more efficacious against these microorganisms. Similarly, among the long chain aliphatic bases, the less strongly basic amines act on gram-positive bacteria for the most part, whereas the gram-negative organisms are more susceptible to the action of the stronger bases such as guanidines and quaternary amines.[29]

Although it might be supposed that the specificity of disinfectants is an undesirable quality and that a universal disinfectant would possess great advantages, it will be apparent on second thought that specific toxicity is often highly desirable. For example, a compound which is equally effective in the destruction of both bacteria and tissue cells could not be used to advantage in the disinfection of the mucous membranes.

The Chemotherapeutic Drugs. The phenomenon of specificity also assumes great practical significance as the basis of chemotherapy of infectious disease. Ehrlich's discovery of salvarsan came as a result of a search for a "magic bullet"—a compound strongly germicidal for a given microorganism yet sufficiently non-toxic to the host that it could be injected into the tissues in effective concentrations. The discovery of chemical structures having these properties has been purely a matter of trial and error rather than rational theory, but once discovered, the active portion of the molecule may, of course, be modified to increase efficacy, decrease toxicity, etc. Successful chemotherapy has, in the past, been confined to certain spirochetoses and protozoan infections, the compounds showing activity including the arsenicals, compounds of antimony and of bismuth, certain dyes such as trypan red and the flavines, synthetic compounds such as atabrine and plasmochin, and certain naturally occurring substances such as quinine. Until relatively recently the bacterial diseases appeared to be, for all practical purposes, resistant to chemotherapy.

With the observation that the azo dye prontosil had marked chemotherapeutic activity in streptococcus infections, and the discovery of the active portion of the molecule, p-aminobenzene sulfonamide (sulfanilamide), a new series of compounds, known as sulfonamides, was found to be highly efficacious in the treatment of many bacterial infections. Many derivatives of p-aminobenzene sulfonamide have been prepared, radicals being attached to the nitrogen of the sulfonamide group, which show different solubilities, degrees of ionization, toxicity, rates of absorption and excretion, associated with differences in chemotherapeutic efficacy.[30]

These compounds are bacteriostatic rather than bactericidal and appear to function *in vivo* by suppressing bacterial multiplication, the invading microorganisms being destroyed by the body defenses, notably through phagocytosis by the cells of the macrophage system (p. 232). They do not affect the bacterial toxins to a significant degree.[31]

[29] Fuller: Biochem. Jour., 1942, 36:548.
[30] *Cf.* Bell and Robin: Jour. Amer. Chem. Soc., 1942, 64:2905.
[31] That is to say, there is apparently no clinically important effect; however, *cf.* Carpenter and Barbour: Proc. Soc. Exp. Biol. Med., 1939, 41:354; Hutner and Zahl: Science, 1942, 96:563.

Another group of bacteriostatic substances, some of which are effective chemotherapeutic agents, are produced by microorganisms and are termed antibiotic substances. These are discussed elsewhere (p. 128).

The Mechanism of Antibacterial Action. As indicated earlier, the differentiation of bactericidal and bacteriostatic activity is essentially one of convenience based on the concentration differential of the active substance, *i.e.*, when the difference between the two end points is great the compound is regarded as bacteriostatic, but when it is very small the bacteriostatic zone is so limited as to appear unimportant and the term bactericidal is applicable. With respect to the mechanism underlying the observed effect, then, no differentiation between the two need be made. It is self-evident that a substance showing antibacterial properties produces its effects through an interruption of essential physiological processes of the bacterial cell. In some instances the effect is relatively non-specific, as in the denaturation of constituent protein,

sulfanilamide   *sulfapyridine*   *sulfathiazole*

sulfadiazine   *sulfapyrazine*   *sulfaguanidine*
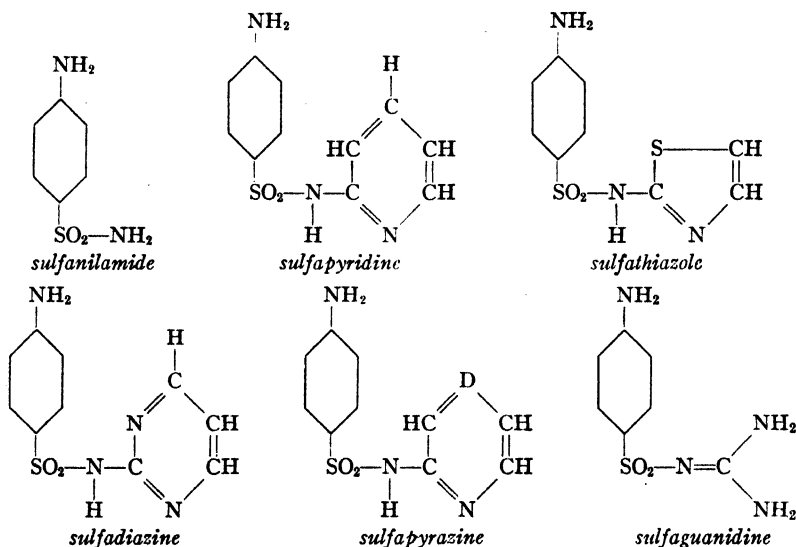
and in others highly specific in that some particular metabolic reaction is affected. There is some tendency to differentiate the former as a general protoplasmic poisoning, but this is more a matter of convenience than an expression of fundamental difference, for a continuous series of degrees of specificity, so to speak, may be set up through which the general toxic effects grade imperceptibly into the inhibition of a single enzyme system. On the other hand, a general distinction may be made between an ionic interchange with the formation of un-ionized complexes with acidic and basic groups of the protoplasm, and a competitive inhibition of enzyme reactions by structurally related analogues of some component of the system. Present knowledge of the mechanism of antibacterial action is based in part on reversal and in part on the antagonistic inhibition of metabolic reactions, both interpreted on the basis of the physiology of the bacterial cell.

Reversal of Antibacterial Activity. Bacteria exposed to the action of a number of bactericidal substances and apparently killed may be "revived" by neu-

tralization of the activity within a short time after exposure. The revival of bacteria presumably dead after treatment with mercuric chloride by treatment with hydrogen sulfide or thiols such as thioglycollic acid, glutathione, and the like has been known for many years. Similarly, bacteria apparently killed with hypochlorite may be revived by inactivating the chlorine with sodium thiosulfate, and the use of thiosulfate solutions for dilution in titration of bactericidal activity of the hypochlorites to neutralize bacteriostatic concentrations carried over into subcultures is a common procedure. Bacteria treated with phenol survive if the phenol is removed by adsorption on charcoal or treatment with ferric chloride.[32] The effects of treatment with dyes such as acriflavine or with cationic detergents may be neutralized with survival of the bacteria by high molecular weight anions,[33] or in the case of dyes, more simply by shifting the pH so that the dye-protoplasm complex is no longer un-ionized, provided that the shift is within physiological limits. In the case of bactericidal concentrations of the compound, it is not clear how far the bactericidal action can be allowed to go and yet permit revival. In many instances it is probable that it has not gone far, perhaps little beyond a preliminary sorption. When the compound is in bacteriostatic concentration only, of course, the cells remain alive for relatively long periods and multiply as soon as the inhibition is removed by neutralization or simple dilution.

Competitive Inhibition. Competitive inhibition of enzyme reactions in which a structural analogue of the substrate competes with the substrate for the enzyme is well known. One of the simplest examples is the inhibition by malonic acid of the oxidation of succinic acid to fumaric acid catalyzed by succinic dehydrogenase in the presence of a hydrogen acceptor. Malonic acid differs from the substrate in that it is a 3- instead of a 4-carbon dicarboxylic acid.

If such a reaction is an essential part of the metabolic processes of a bacterium, the inhibitor exhibits antibacterial activity. The immense practical significance of this was pointed up in the observation by Woods in 1940 that the antibacterial activity of sulfonamide is specifically inhibited by p-aminobenzoic acid, a compound that is required as a bacterial vitamin by a number of organisms, including *Clostridium acetobutylicum, Acetobacter suboxydans* and some strains of the diphtheria bacillus. The close similarity in the structure of p-aminobenzoic acid and p-aminobenzene sulfonamides will be obvious from the accompanying structural formulae. That p-aminobenzoic acid is required by some bacteria suggests that it is involved in some essential metabolic reaction, and it was suggested by Woods and by Fildes that the action of the sulfonamides on bacteria is the result of a competition of these compounds with an essential bacterial metabolite, p-aminobenzoic acid. This hypothesis is known as the *Woods-Fildes theory.* It is of particular significance since it is the first rational theory of chemotherapy and, like any sound theory, allows prediction and test of prediction.

This explanation of chemotherapeutic activity is now supported by an impressive amount of evidence; and it appears highly probable that it is sub-

[32] Flett, Haring, Guiteras and Shapiro: Jour. Bact., 1945, 50:591.
[33] McIlwain: Biochem. Jour., 1941, 35:1311;Valko and DuBois: Jour. Bact., 1944, 47:15.

stantially correct.[34] For example, the bacteriostatic action of a wide variety of derivatives of sulfanilamide is nullified by *p*-aminobenzoic acid and related compounds which will give it on hydrolysis. The relationship between the antagonist and drug is a quantitative one; the ratios usually reported vary from 1 : 1000 to 1 : 26,000 but are considerably nearer unity if the presence of antagonist in the test medium and the ionic concentration of the drug are taken into consideration.

It may be noted that, as a corollary, specimens of blood, urine or other body fluids taken from an individual undergoing sulfonamide therapy may contain sufficient amounts of the drug to inhibit bacterial growth when they are cultured. The inhibiting effect may be avoided by including *p*-aminobenzoic acid in the culture medium; a concentration of 5 mg. per 100 ml. is adequate.

With generalization of the idea of competitive inhibition of essential metabolic reactions, antagonisms to other bacterial vitamins may be predicted and tested. This has been done, especially by McIlwain. It has been found that the modification to produce an analogue may vary and may include the replacement of a carboxyl group with a sulfonic acid or sulfonamide radical, or by a ketone, by the substitution of one atom for another in the ring system of aromatic metabolites, the replacement of alkyl side chains of ring systems with halogens, etc. For example, sulfonic acid and sulfonamide analogues of nicotinic acid and pantothenic acid have been prepared by substitution of $-SO_3H$ or $-SO_2NH_2$ for a carbonyl group and have been found to be specifically antagonistic and inhibitory. Similar results have been obtained with pyrithiamine, the pyridine analogue of thiamine. The reverse kind of experiment has been carried out also. Iodinin, the di-N-oxide of a dihydroxyphenazine, is a pigment produced by *Chromobacterium iodinum* which inhibits the growth of certain bacteria. The inhibitory effect can be nullified by certain anthraquinones and naphthaquinones, from which it may be inferred that quinones play some part in bacterial metabolism, possibly in the transport of hydrogen. As yet none of the analogues tested have had practical chemotherapeutic activity though some effect may be observed; large doses of thiopanic acid (pantylyltaurine, an analogue of pantothenic acid) protects rats against lethal doses of hemolytic streptococci.

It may be noted in passing that the generalization of this theory seems to remain valid when carried over to higher organisms. Thus, symptoms of vitamin deficiency may be produced in experimental animals by feeding an analogue of the vitamin, *e.g.*, pyrithiamine, glucoascorbic acid, iso-riboflavin and the phenazine analogue of riboflavin, β-acetyl pyridine (an analogue of nicotinic acid).

The mechanism of competitive inhibition is in most cases more complex than here indicated. It is frequently assumed that analogues compete with metabolites acting as coenzymes, but in general the evidence indicates that the competition occurs when the metabolite acts as a substrate rather than a coenzyme. As a substrate, the metabolite is synthesized into a more complex molecule which functions as a coenzyme, or it may be degraded prior to synthesis. Thus, *p*-aminobenzoic acid is a part of folic acid and competition

[34] See the reviews by Wooley: Advances in Enzymology, 1946, 6:129; Physiol. Rev., 1947, 27:308; Hotchkiss: Ann. Rev. Microbiol., 1948, 2:183.

apparently occurs in the synthesis of the vitamin. Similarly, pyrithiamine does not compete with thiamine pyrophosphate when the latter functions as co-carboxylase, but rather competition appears to occur in the synthesis of the coenzyme from thiamine. It was early assumed that, if a compound is required by certain bacteria, it is an essential metabolite for other bacteria also which do not show the requirement because they are able to synthesize it; it would appear to follow that a drug could compete with a vitamin either preformed or after synthesis by the microorganism. It is not quite so simple as this, however, for it has been found that bacteria which do not require thiamine, for example, are able to split pyrithiamine into pyrimidine and pyridine in the same way that thiamine is split into pyrimidine and thiazole components, and that organisms which cannot synthesize thiamine also lack the ability to split pyrithiamine and are susceptible to its inhibitory activity. Such somewhat indirect relationships to the better known metabolic reactions of bacteria probably account, to some extent at least, for evidence which appears to be inconsistent with a hypothesis of direct competitive inhibition.

The Effects of Antibacterial Compounds. The ways in which antibacterial substances affect the cell so as to produce either irreversible changes in the cell organization resulting in death, or an inhibition of processes essential to reproduction, vary. Available evidence indicates, as might be expected, that a variety of effects may be produced which will give the observed results. Thus a general oxidation of cell substance by oxidizing agents, a saturation of thiol groups of constituent proteins with heavy metals, or a generalized denaturation of protein by, for example, the substitution of halogens such as iodine in the ring structures of aromatic amino acids, obviously alters the organization of the cell on a macromolecular level to such an extent that normal metabolism cannot continue. Bactericidal substances of the phenol group apparently act in this way in that phenol is surface-active and by orientation of the hydroxy groups reacts with free amino groups of the cell proteins.[35] Even those substances which appear to be general protoplasmic poisons, however, may show some specificity of action; there is evidence that chlorine, for example, first affects a triosephosphate dehydrogenase system.[36]

Or the effect may be somewhat more localized as in the case of the basic dyes which react with nucleoprotein and whose action, at least that of acriflavine, is antagonized by nucleotides; it is not unreasonable to suppose that the hereditary mechanisms of the bacterial cell are made up of nucleoprotein and their inactivation by the formation of un-ionized dye-protein complexes inhibits proliferative processes. Similarly, the surface-active detergents are antagonized by lipids, *e.g.*, the anionic detergents by cephalin, and there is some reason to believe that they alter the permeability of the cell wall to such an extent that the cell dies.

Interference, by competitive inhibition or otherwise, with specific metabolic reactions is, perhaps, more obvious when these are essential to the metabolism of the cell. Such metabolic reactions may be the energy-yielding oxidations of the respiratory process and, as indicated elsewhere, the bactericidal efficacy of many compounds may be estimated as accurately by inhibition of oxygen

[35] See Fogg and Lodge: Trans. Faraday Soc., 1945, 41:359.
[36] Green and Stumpf: Jour. Amer. Water Works Assn., 1946, 38:1301.

7

uptake in respirometers as by methods using death as an end point. Many of the important antibacterial substances, however, do not appreciably affect the respiratory mechanisms and there is reason to believe, in some cases at least, that they interfere with synthetic mechanisms; for example, there is some evidence that the sulfonamides interfere with synthesis. Other compounds, such as azide and dinitrophenol, actually increase oxygen uptake, in the presence of carbohydrate substrates, but the increased oxidation is due to a prevention of assimilation of carbon by the cell. It will be clear that, while information as to the precise point of attack of antibacterial substances on cell metabolism is far from complete, a variety of mechanisms is not only possible but operative.

Potentiating and Interference Effects. As yet unexplained are the results of mixing antibacterial substances. In some cases, of course, the antibacterial activity of two substances is simply additive. In others, however, the effect is potentiating in that the activity of the combined substances is greater than a purely additive activity, and the activity of some compounds may be enhanced by mixture with inactive ones. Such potentiating effects are observed in the combination of penicillin (p. 131) with gramicidin (p. 128) or sulfonamides, or in the combination of sulfonamides with azochloramide. The activity of sulfonamides may be enhanced by mixture with inactive substances such as carbamates, urea, asparagin, and the like. In still other instances, notably the incorporation of bactericidal substances with soaps, the combined effect is less than an additive one.

Factors Influencing Disinfection. The process of disinfection or bacterial death is often, in part at least, a chemical reaction and is, therefore, subject to a variety of influences which affect the velocity of such reactions. The most important of these influences is the concentration of the reacting substances, *i.e.*, the concentration of disinfectant and the numbers of bacteria present. The *effective* concentration of disinfectant is, in turn, dependent upon two other factors, first, the presence of moisture, which makes possible coagulation by heat, and ionization of the bactericidal salts, and acts as a solvent and suspending medium in which there may be intimate contact between the disinfectant and the microorganism; and, second, the presence of extraneous organic matter. Many chemical disinfectants act through a combination with the protein of the cell and, if extraneous organic matter is present, will, of course, react with this inert material, thereby reducing the effective concentration. Disinfectants vary widely in the degree to which their bactericidal properties are affected by organic matter. The salts of the heavy metals are rapidly precipitated by organic material, while compounds such as phenol and the cresols are only slightly affected. The rate of destruction by heat is also affected by the presence of organic matter—organisms embedded in a mass of fecal material, for example, are protected from heat for a short time. The process of disinfection by germicides or by heat is influenced by temperature, the velocity of the reaction increasing with rise in temperature. The *p*H likewise influences the rate of bacterial destruction not only by heat but by many chemical compounds, the velocity, in general, being least at neutrality and increasing with increase in acidity or alkalinity. A number of other factors such as the presence of salts, etc., affect the rate of disinfection but generally not sufficiently to be of practical importance.

From the practical point of view, the time of exposure of bacteria to a given disinfectant is of considerable significance and, of course, bears an inverse relation to the rapidity of killing. The time allowed for the destruction of bacteria is determined not only by the factors discussed above but also by the kind of bacteria that are to be killed. In certain cases the specificity of a disinfectant may be so marked that it must be taken into consideration. For example, the relative atoxicity of hypochlorite for the tubercle bacillus referred to above precludes its use in the disinfection of tuberculous sputum. Bacterial spores are much more resistant to heat and chemical disinfectants than are the vegetative cells, and considerably more time must be allowed for their destruction. The vegetative cells of some bacteria may be somewhat more resistant than those of others, but, for the most part, such differences are too small to be of practical significance.
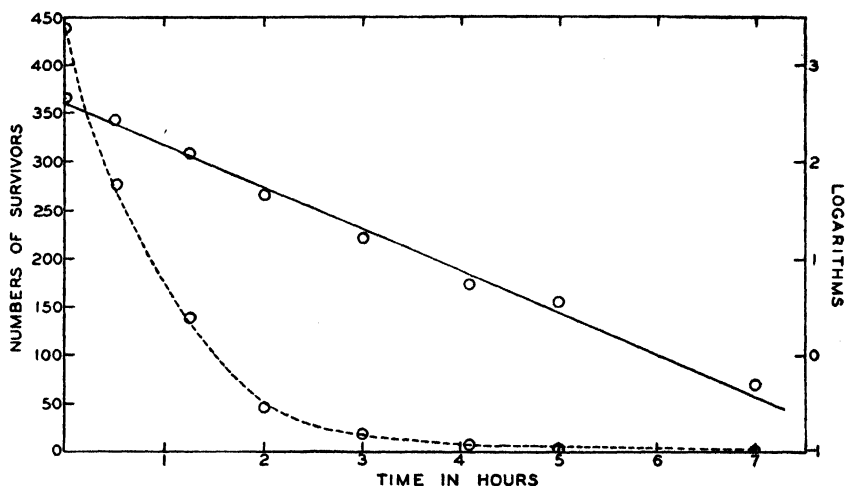
Fig. 23. Death rate of anthrax spores treated with 5 per cent phenol. The dotted line is the arithmetic plot and the solid line the logarithmic plot. The negative logarithm was obtained by taking samples of several times the unit volume. (After Chick: Jour. Hyg., 1908, 8:92.)

The Dynamics of Disinfection. Quantitative studies on the rate at which bacteria are killed by lethal agents have indicated that in many instances the organisms die at a logarithmic rate, *i.e.*, if the logarithms of the numbers of viable organisms are plotted against time, the points tend to fall on a straight line. This phenomenon has been observed in the death of both spores and vegetative cells under the influence of chemical disinfectants or moist heat and also occurs in the death of bacteria in old cultures. The velocity of the reaction, the slope of such a line, depends upon the concentration and kind of disinfectant, the nature of the organisms—whether spores or vegetative cells— and other factors which influence the process of disinfection. This logarithmic rate likewise describes the course of a monomolecular reaction, and this fact has led some to conclude that bacterial death is a monomolecular chemical reaction. Although the killing of bacteria by some disinfectants is undoubtedly

a chemical process, as for example the precipitation of constituent protein as proteinates of heavy metals, the evidence does not justify the conclusion that the reaction is monomolecular. The fallacy is the old one of confusion of correlation and causality under the slightly different guise of description and mechanism. Experimental evidence has indicated that while such semilogarithmic plots may often be fitted with a straight line, others are best fitted by sigmoid curves, the death rate being more rapid in the beginning in some cases and more rapid at the end in others or even highly irregular. Some workers[37] have concluded that one of the more important determining factors is a graded biological resistance of the cells in the bacterial population, an explanation that is supported by general biological considerations. Undoubtedly the phenomenon of bacterial death is a complex of interrelated factors whose admixture with the mechanics of the mass law results in the parameters determined by mathematical means. Of practical importance, however, is the fact that in disinfection by chemicals and by heat there is a minority of cells, possibly more resistant, that survive long after the majority have perished and that must be destroyed in order to obtain complete sterilization.

The Standardization of Disinfectants. The relative bactericidal efficiency of the chemical disinfectants is a point of considerable practical importance. The value of a quantitative method for the determination of the killing power of germicides was recognized early in the development of bacteriology, and experimental investigation led to the establishing of a standardized technique which made possible the determination of the bactericidal power of a given chemical compound relative to that of phenol. The numerical value so determined is termed the *phenol coefficient* and is presumed to indicate whether, and to approximately what extent, the unknown is a better or poorer germicide than phenol. Later methods of standardization have grown out of this procedure. The phenol coefficient of a disinfectant used against the typhoid bacillus is calculated as follows:

Divide the greatest dilution of the disinfectant capable of killing S. *typhi* in ten minutes but not in five minutes by the phenol dilution which so kills this, and divide these figures one into another. In order not to convey a false idea of the accuracy of the method the coefficient is calculated to the nearest 0.1 point if under 1, to the nearest 0.2 point if between 1 and 5, to the nearest 0.5 point if between 5 and 10, and to the nearest 1.0 point if between 10 and 20.

For example, if a 1:90 dilution of phenol kills in 10 minutes but not in 5, and a 1:100 dilution does not kill in 10 minutes, the former is taken as the end point; and if a 1:350 dilution of the unknown disinfectant kills in 10 minutes but not in 5, while 10 minutes' exposure to a 1:400 dilution does not kill, the end point is the 1:350 dilution. The phenol coefficient is the ratio of these two, *i. e.*, 350/90 = 3.89 or 3.9, and the unknown has, by this method, 3.9 times the killing power of phenol. The conditions regarded as standardized in the United States, the FDA phenol coefficient, have been defined by the Food and Drug Administration.[38]

The effect of extraneous organic matter on the bactericidal power of a disinfectant is commonly taken into consideration by carrying out the test with and without added organic matter. Three per cent of dried fecal matter or dried yeast may be added to the bacterial suspension or the organisms may be

[37] Knaysi: Jour. Inf. Dis., 1930, 47:293.
[38] United States Department of Agriculture Circular No. 198. 1931.

BACTERICIDAL ACTIVITY OF DISINFECTANTS*

Compound	Type	Phenol Coefficients†						Skin Sterilization‡		
		Staph. aureus		Strep. viridans		Bact. coli		Compound	Number of skin grafts	Per cent sterile
		Water	50% serum	Water	50% serum	Water	50% serum			
Merphenyl nitrate (1 : 1500 aqueous)........	R§ A¶	0.409 614	0.133 200	0.250 375	Not done	0.272 408	0.044 66	Merphenyl nitrate tincture 1 : 1500	100	0
Merthiolate (1 : 1000 aqueous)...	R A	0.188 188	0.077 77	0.031 31	Not done	0.043 43	0.03 30	Merthiolate tincture 1 : 1000	30	6
Metaphen (1 : 500 aqueous).......	R A	0.375 687	0.155 77.5	0.80 400	Not done	1.26 630	0.07 35	Metaphen tincture 1 : 200	50	80
Mercurochrome (1 : 50 aqueous)...	R A	0.040 2.0	0.022 22	0.025 1.25	Not done	0.040 2.0	0.02 20	Mercurochrome tincture 1 : 50	40	10
Hexylresorcinol (1 : 1000 aqueous)	R A	0.063 63	0.022 22	0.062 62	Not done	0.043 43	0.02 20	Phenol 2% aqueous	30	0
Mercuric chloride (1 : 1000 aqueous).......	R A	0.175 175	0.020 20	0.125 125	Not done	0.150 150	0.020 20	Alcohol acetone solution	30	0
Iodine, tincture (7% in alcohol-KI solution).........	R A	20 286	0.355 5	8 112	Not done	10 143	0.35 5	Iodine, tincture 7%	40	80

* Selected from the data of Clampit, Campbell and Reames of the University of Chicago.
† Determined on basis of killing in five but not in ten minutes.
‡ The disinfectant is applied to an area of shaven skin, a piece of which is then excised and cultured.
§ Relative phenol coefficient determined by dilution of the disinfectant as supplied (concentration in parentheses).
¶ Absolute phenol coefficient based on the pure compound.

suspended in 50 per cent serum. It is important to recognize that bacteria differ considerably in their resistance to phenol, staphylococci, for example, being much more resistant than the typhoid bacillus, so that in strict accuracy it is necessary to specify "typhoid phenol coefficient," "pneumococcus phenol coefficient," etc.

It is open to question as to whether compounds whose structure and chemical and germicidal activity differ greatly from that of phenol may legitimately be compared with this standard disinfectant. In recent years some workers have departed from the standard procedures, altering them to suit individual requirements. The phenol coefficient of an alcoholic solution of a compound insoluble in water obviously has little value, as has that determined on an acid or alkaline solution of a compound only slightly soluble at neutrality.[39] The simple statement that a given germicide has such and such a phenol coefficient has, then, little meaning.

Even assuming, however, that the standard phenol coefficient procedure be rigidly adhered to, the test itself is open to serious criticism. The end point to be determined is, clearly, sterility—all the test organisms must be destroyed. The quantitative studies discussed above have shown that although destruction may proceed at a regular and rapid rate for a time, the asymptotic tendency of the survival curve indicates that sterility or complete destruction is not a desirable end point in that it is one that is difficult to determine with a reasonable degree of accuracy. The practical consequences of the use of this end point appear in the form of aberrant results when the time variable is altered. If the end point be taken as sterility in two and one-half minutes as in the original Rideal-Walker method, the phenol coefficient of $HgCl_2$ is somewhat more than 2, but if the time taken is thirty minutes as in the Chick-Martin modification, $HgCl_2$ has a phenol coefficient of 550.[40]

Furthermore, the phenol coefficient test does not take into consideration the temperature coefficient of the disinfectant under examination, which differs with the test organism,[41] nor does it measure the effects of changes in concentration. A rise in temperature increases the activity of phenol and similar compounds much more than that of the salts of heavy metals; doubling the concentration of phenol increases its bactericidal activity approximately 64 times, while a similar increase in the concentration of $HgCl_2$ only roughly doubles its activity.

It is generally agreed that the rate of killing, *i.e.*, the reaction velocity constant, is a much more accurate measure than any value whose derivation depends upon a sterility end point. The relation between concentration of disinfectant and time required for killing is an exponential one and the reaction velocity constant, k, is given by the expression:

$$k = C^n t$$

when C is the concentration, n a constant characteristic for each disinfectant, and t is time. The temperature coefficient may, of course, be determined ex-

[39] For a discussion of the limitations of the phenol coefficient see Reddish: Ind. Eng. Chem., 1937, 29:1044.

[40] Chick: Jour. Hyg., 1908, 8:92; *ibid.*, 1910, 10:237; *ibid.*, 1912, 12:414.

[41] *Cf.* Tilley: Jour. Bact., 1942, 43:521.

perimentally for each disinfectant. It will be clear that this kind of characterization of the bactericidal activity of a given compound is much more informative than the usual type of phenol coefficient, no matter how precisely the conditions of the test are defined. It is not, however, generally used for routine work.[42]

The practical value of a disinfectant is not always indicated by tests made under the controlled conditions of the laboratory. Hydrogen peroxide, for example, may give a quite respectable phenol coefficient but, when applied to an abrasion, is so rapidly decomposed under the influence of tissue catalase that its germicidal powers are almost immediately exhausted.

On the other hand, a given disinfectant may be so highly toxic for tissue cells that it has no practical value. Salle and his associates[43] have proposed a "toxicity index" which takes into consideration not only the germicidal activity of a compound but its toxicity for tissue as well. For example, Witlin[44] determined the concentration of bactericidal substances lethal for the chick embryo and calculated a toxicity index by using the concentration of the bactericide in grams per milliliter killing the test organism, *Staphylococcus aureus*, in ten minutes but not in five as the numerator, and the chick embryo MLD in grams for the denominator. Representative values were: phenol $(1:20)$—1.18; $HgCl_2$ $(1:1000)$—0.62; tincture of iodine—0.044; sodium hypochlorite—1.39; mercurochrome $(1:50)$—13.3; and values ranging as high as 9.1 for various organic mercurials. Spaulding and Bondi[45] have developed an infection-prevention toxicity test in which the tip of a mouse tail is contaminated with bacteria, the tail dipped into the solution of disinfectant, and then the tip is removed and placed in the peritoneal cavity. The highest dilution of the disinfectant protecting 50 per cent of the mice is taken as the numerator, and the greatest concentration allowing survival (from poisoning) of the test animal is taken as the denominator, to give a ratio or infection-prevention-toxicity index. Such toxicity indices are of considerable practical importance in the case of skin disinfectants. There is as yet no standard method for their testing.[46]

The whole question of the evaluation of disinfectants is beset with many difficulties, both theoretical and practical, and to date remains an open one to which no entirely satisfactory answer has been supplied.

[42] For applications of this approach see, for example, Withell: Jour. Hyg., 1942, 42:124, 339; Irwin: *ibid.*, 1942, 42:328; it has also been studied extensively by Jordan and Jacobs: Jour. Hyg., 1944, 43:275, 363; *ibid.*, 1945, 44:210; Ann. Appl. Biol., 1945, 32:221; Jour. Hyg., 1946, 44:243, 249, 421.

[43] Salle, McOmie and Schechmeister: Jour. Bact., 1939, 37:639.

[44] Witlin: Proc. Soc. Exp. Biol. Med., 1942, 49:27.

[45] Spaulding and Bondi: Jour. Inf. Dis., 1947, 80:194.

[46] *Cf.* Report of the Council on Pharmacy and Chemistry: Jour. Amer. Med. Assn., 1943, 121:593.

BACTERIAL HEREDITY AND VARIATION

Before the development of methods of isolating bacteria in pure culture many observers regarded these organisms as members of a markedly homogeneous group, possibly of but a single species, which showed a high degree of morphological variation. The interconversion of coccus, bacillary and spiral forms was considered to be of common occurrence and, in consequence, the observed morphological differences among the bacteria were held to be of no significance. This doctrine, designated as *pleomorphism,* reached its height in 1877 with Nägeli, who postulated but a single species to which all bacteria belonged. It should be noted that the term pleomorphism included not only morphological variation but equally facile alterations in virulence; the innocuous hay bacillus (*Bacillus subtilis*), for example, presumably could change suddenly into the highly virulent anthrax bacillus.

The development of pure culture techniques by Koch and others swung the pendulum to the other extreme, and the doctrine of *monomorphism* became predominant. It was held by Koch and other extremists, whose camp the great majority of bacteriologists soon joined, that a given bacterial species existed in one and only one form, and that aberrant forms were either evidence of contamination of the pure culture or were the so-called involution or degenerative forms which were dead or dying and therefore had no significance. This monomorphism was confined largely to bacterial form and, although abrupt changes such as the conversion of the hay bacillus to the anthrax bacillus were denied, alteration in the virulence of pathogenic bacteria was not regarded as inconsistent with an absolute constancy of form.

Despite the currency of this extreme monomorphism, evidence began to accumulate which indicated that, though most bacteria showed a remarkable constancy of form, morphological and physiological variation of these organisms is not uncommon. The last thirty or forty years have seen the development of an extensive literature on this subject. Much of the evidence which has accumulated is of just sufficiently inconclusive character that widely different interpretations may be made. Contemporary workers fall into two groups in this respect: the conservatives, a group which includes the majority of workers, who still adhere to the doctrines of Koch and his school in a modified form and who accept the well established variations as observed facts whose full significance is as yet not apparent; and the radicals, who profess to see evidences of complex life cycles, conjugation, sexual reproduction and kindred phenomena in the reported observations. It is not improbable that a sound interpretation lies somewhere between these views.

The biological significance of observed variations and their relation to similar phenomena occurring in the higher forms of life are difficult to assess. The difficulties stem in part from a strong tendency to regard bacteria as somehow "different" from other living organisms. This inherently sterile concept has arisen partly because of the apparent absence of a morphologically discrete nucleus in the bacterial cell, partly because the characteristics of bacteria which vary are generally not directly comparable to the genetically determined characters which are studied in the higher organisms, and partly because the bacteria are subject to the influence of unique environmental factors.

While it seems not unlikely that the chromatinic bodies described by Robinow (p. 51) represent an organized nucleus in the bacterial cell, there is as yet no indication of an intranuclear differentiation into structures analogous to chromosomes. Present evidence suggests that nuclear division, if the division of chromatinic bodies may be so regarded, is not as complex, at least morphologically, as the process of mitosis. While there is evidence, as will appear, of a spatial orientation of hereditary determinants, no corresponding morphology is as yet apparent, and the cytological techniques so essential to the understanding of the genetics of higher organisms have not been applicable to the bacteria.

The differential characters which serve to distinguish bacteria from one another are, in general, of a greater degree of fineness than those which the biologist is accustomed to use. The bacteriologist is concerned with characters not so gross as the red and white eyes of Drosophila but which might be considered analogous to the individual enzyme systems operative in the formation of red and white eyes. When such characters have no counterpart among those known to be of fundamental significance to the higher organism, and most if not all of them as yet have not, it is extremely difficult to judge whether they are of deep-seated importance to the cell or whether they are biologically trivial. Loss of virulence by a pathogenic organism, while of great practical importance to its prospective host, may be of only minor significance to the bacterium. Analogies are, then, difficult to draw and are always of doubtful validity.

Not only are the environmental factors which affect the bacterial cell to some degree peculiar to unicellular organisms of minute dimensions, but the essential obscurity of the bacterial environment is, to no small degree, a consequence of the limits of human imagination. Some aspects of this bacterial environment are known. For example, the tremendously exaggerated effect of surface tension on objects as small as bacteria must profoundly influence bacterial form. The continuous molecular bombardment to which these organisms are subject and to which, because of their small size, they respond with the dancing motion of brownian movement, may be a factor which influences their structure and rigidity of form. The predominance of interfacial phenomena in the bacterial world of near-molecular dimensions undoubtedly contributes to its general character. There is evidence, for example, that hydrogen ion activity and reducing intensities may be quite different at interfaces from what the observer of gross phenomena assumes them to be. The environment to which these and undoubtedly other phenomena lend

character is, clearly, an abstract one, and the interpretation of morphological or physiological changes taking place under such circumstances is, to say the least, difficult.

From these considerations it is easy to understand how bacteria can come to be regarded as "different" without real justification for such a belief. It cannot, then, be emphasized too strongly that, so far as is known, bacteria do not differ in any essential way from other living cells and any interpretation of variation or other phenomena must rest on a sound biological foundation.

Before discussing the kinds of bacterial variation which are observed, two concepts fundamental to the general problem must be considered. One of the most important of these is that of the "normal" morphological or physiological state of a bacterium. The anthropocentric tendencies of the human mind
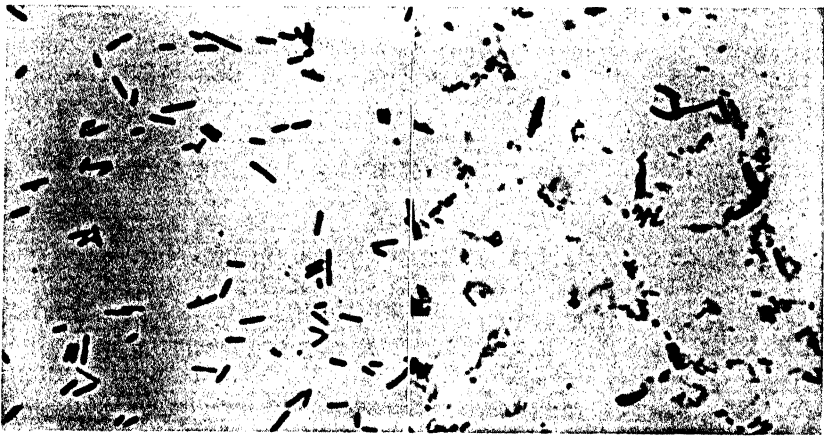
Fig. 24. The morphology of Rhizobium. Left, a smear from a pure culture on laboratory media; note the regularity of the "normal" bacillary morphology. Right, smear from a clover root nodule showing swollen, branched and coccoid forms, the so-called bacteroides. Fuchsin; × 1050.

stimulate the belief that the normal form and functions of a bacterium are those which are manifest in culture on the ordinary laboratory media. Certain evidence suggests, however, that the term normal is but a relative one. The symbiotic nitrogen-fixing organisms show a relatively constant "normal" morphology on the usual laboratory media, but in the root nodule they are found in a variety of bizarre forms designated as bacteroides (Fig. 24). Which, then, may be considered to be the "normal" form, that observed in the laboratory or that shown by the organisms in what might be regarded as their natural habitat? The physiological differences between bacteria in pure culture and those in the mixed cultures occurring in nature have been discussed in Chapter 4. Clearly, then, the term normal, although an extremely useful one, cannot be taken to have real meaning.

The second concept is that of bacteria in terms of populations. The existence of bacteria as population groups composed of enormous numbers of individuals and a rapid rate of cell division results in a compression in both time and space of biological processes. The resulting exaggeration of variabil-

ity of the bacteria as compared to that of other living organisms, coupled with the selective effect of population pressure as numbers approach a limiting density, undoubtedly contributes in very large part to the apparent flexibility in the response of bacteria to environmental conditions. The importance of the population aspect of bacteria has only recently become more generally recognized,[1] though inter- and intraspecies relationships at the population level of other organisms constitute a large part of ecology.

Here bacterial variation will be considered under two general heads, the kinds of variation observed, and the mechanisms operative in such variation.

OBSERVED VARIATIONS OF BACTERIA

Bacteria have been found to vary, sometimes widely, in all the characteristics made use of in their differentiation and identification. These include morphology, both macroscopic and microscopic; physiological properties, including ability to produce disease; and immunological character. Changes may appear suddenly, *i.e.*, in a single subculture, or they may become apparent only gradually as in aged cultures or over many transplants or animal passages and some hundreds of generations. The rapidity of appearance, however, cannot be taken to have fundamental significance and, in fact, in many instances is subject to experimental manipulation. Variants may appear apparently spontaneously, or may be seemingly induced by making the culture medium mildly and specifically toxic, as by the inclusion of lithium chloride, antiserum, antibacterial substances and the like, or by making it selective so that the expected variant is given enhanced survival value as, for example, by including a sugar in the medium in attempts to isolate fermenting variants of a non-fermenting parent strain. The relative importance of the characters subject to variation is by no means clear, and some may be biologically trivial while others are a reflection of deep-seated changes within the cell. It may be emphasized that relative biological importance is not necessarily related to practical importance.

MORPHOLOGICAL VARIATION

Variation in the morphology of bacteria may be considered under two general heads, colonial morphology or that of masses of cells, and the morphology of individual bacterial cells.

Bacterial Dissociation. A remarkable type of bacterial variation, whose most obvious outward manifestations is a change in the type of colony formed on semisolid media, was observed by Baerthlein in Germany (1918), Arkwright in England (1921) and de Kruif in the United States (1921). The phenomenon was termed by de Kruif bacterial or microbic dissociation, a term which has, in spite of certain undesirable features, been generally adopted.

The ordinary laboratory culture, particularly old broth cultures, when plated out on an agar medium develops into two kinds of colonies; one, smooth (S), round, convex and shining; the other, rough (R), irregular, flattened and wrinkled. In addition to these obviously different extreme colony types (Fig. 25), all degrees of intermediate (SR and RS) types may usually be found. The transformation of the S form, usually regarded as the

[1] See Braun: Bact. Rev., 1947, *11*:75.

"normal" into the R type is, as indicated by the presence of intermediate types, probably a gradual process. The smooth form of the great majority of bacteria, while breeding true for a number of test tube generations, shows a constant tendency to change to the rough type; one of the consequences of this tendency is that most stock laboratory cultures contain a large propor- tion of roughs, and sometimes such cultures are completely lacking in smooth forms.

A third kind of colony, the M or mucoid, is also commonly recognized, characterized by a slimy, mucus-like consistency and appearance. The mucoid character may be associated with the S or R colonial types to give mixed or intermediate forms, but it is thought by many that S is intermediate between M and R and that the succession is $M \rightleftharpoons S \rightleftharpoons R$. In individual cases other colonial forms may be observed; the hemolytic streptococci, for example, occur
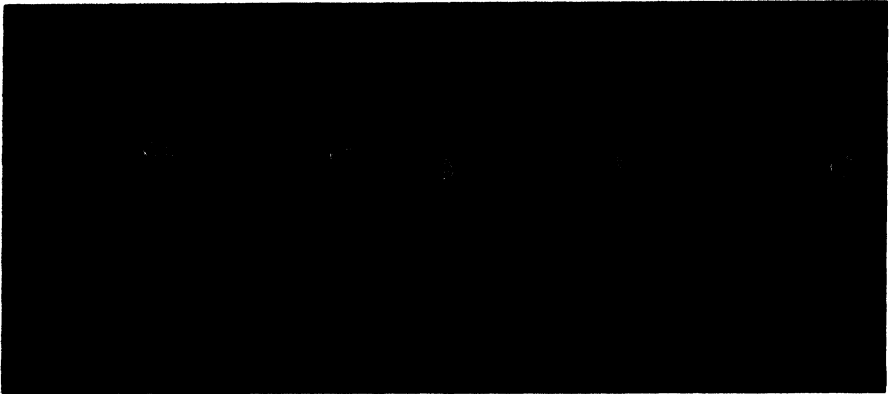
Fig. 25. Smooth and rough colonies of *Bact. typhosum;* × 3. Culture on nutrient agar inoculated from an old broth culture.

in a form designated "matt" by Todd and the British workers which has a ground-glass appearance when viewed by reflected light.

The transformation of S to R, though occurring in ordinary cultures, may be hastened by a number of procedures, such as the inclusion of lithium chloride or anti-S immune serum in the medium in which the organisms are cultured. Smooth cultures lysed by bacteriophage, a filterable virus parasitic on bacteria (Chapter 38), give rise when incubated to a secondary growth which often consists entirely of R forms. The change from R to S is con- siderably more difficult, and the rough forms show little tendency to revert under ordinary cultural conditions. The reversal may, however, be accom- plished in most cases, the best test tube method being the cultivation of the R form in the presence of anti-R immune serum. Reversion of the R type may also be brought about by animal passage.

The bacteria which make up the smooth and rough colonies differ from one another in a number of characteristics, some of which are usually re- garded as of deep-seated biological significance. These are most conveniently considered in the accompanying outline form.

Smooth (S) Type	Rough (R) Type
(1) Broth cultures uniformly turbid	(1) Sedimental deposit in broth cultures; supernatant fluid clear
(2) Suspensions in salt solution (0.85 per cent NaCl) stable and remain cloudy	(2) Suspensions in salt solution clump spontaneously and settle out
(3) Flagellated species usually motile	(3) Motility reduced or absent
(4) Capsulated species show capsules	(4) Capsules absent
(5) Somatic and flagellar or type specific antigens present; flocculent agglutination	(5) Only somatic antigens present; agglutination granular in type
(6) Pathogenic species generally virulent	(6) Virulence greatly reduced or absent
(7) Biochemically active	(7) Biochemical activity reduced
(8) "Normal" morphology	(8) Tendency toward abnormal forms

The distinctions between the S and R types as outlined here represent a generalization to which there are a number of exceptions. For example, what is usually regarded as the "normal" form of the anthrax bacillus is the rough form and in this case the organism is virulent in the rough form but avirulent in the smooth form. Similarly, the "normal" virulent form of the hemolytic streptococci is finely granular rather than smooth, and the avirulent form which arises from it has lost its type-specific antigen but is smoother than the virulent form. In spite of these and other exceptions, the differences noted above hold true in general.

Closer inquiry into the differences between S and R bacterial types indicates that the relation between colony form and certain other characters is not in the nature of a casual correlation but rather the result of an intimate association. The association between capsules and virulence has been pointed out previously, as has been the role of the capsule in giving the colony of capsulated bacteria a slimy, viscous consistency. It is not unreasonable to expect, then, that a bacterium which lacks a capsule is also avirulent, or relatively so, and that its colonies will be dry and possibly wrinkled in appearance. The loss of a capsule likewise alters the nature of the bacterial surface; the absence of carbohydrate material allows the lipids present in the cell membrane to determine its character; in consequence, it becomes hydrophobic rather than hydrophilic and the organisms tend to clump and settle out of salt solution or the nutrient broth of a culture. The granular consistency of many of the rough colony types is accounted for, in part, by post-fission movements of the cells. The smooth forms of some of the intestinal bacilli separate after fission and slip to lie side by side with one another. The cells of the rough forms, on the other hand, tend to remain attached after fission with the formation of chains which bend at sharp angles here and there. The development of bizarre shapes by some of the rough forms may possibly be a result of the absence of capsular material and change in the nature of the cell surface. The nature of the association between roughness and reduced biochemical activity is unknown.

It is not improbable that the R form is the better adapted to the highly competitive conditions in the aging culture and the S form is selected against by this environment, thus accounting for the appearance of the R variants in older cultures. Such a situation has, in fact, been shown to occur in Brucella

cultures by Braun.[2] Similarly, the inclusion of antiserum to one dissociative form markedly favors the development of the heterologous form.

The G Colony. Another type of colony, the so-called G type, although apparently seen by earlier observers in 1910 and 1911, was brought to general notice by Hadley and his co-workers. These colonies are very small, 0.05 mm. or less in diameter, and appear to be made up of small cells of diverse morphology. They may be regarded as variants with an inherently lowered metabolic rate which perhaps permits survival under unfavorable conditions.[3]

L Variation. Perhaps related to G colonial variation is the so-called L variation. The normal mode of reproduction of the pleuropneumonia-like organisms and the non-sporulating obligate anaerobic bacteroides includes the formation of greatly swollen cells or large spherical bodies from which bacilli or granular forms are liberated (pp. 540 and 548). The latter give rise to very minute colonies similar to the G colony. This phenomenon has also been reported as occurring in other bacteria such as Proteus, but with somewhat less convincing evidence.

Variation in Cell Structures. As indicated above, colonial morphology is in part a function of the morphology of the individual cells making up the colony, and one of the most important factors in the differential morphology of the S and R variants is the occurrence of hydrophilic polysaccharide on the surface of the cell. As pointed out elsewhere (p. 42), this frequently takes the form of a layer or morphologically demonstrable capsule on the outer surface of the cell.

Capsules. Capsule formation or, from the physiological point of view, the synthesis of capsular substance usually of polysaccharide but sometimes of polypeptide nature, is very common among the bacteria, but a morphologically demonstrable capsule is not always apparent. Capsule formation is dependent in large part on the environment, and the most favorable medium in the case of pathogenic bacteria in which capsule formation is associated with virulence is the body of the susceptible animal. The anthrax bacillus forms a heavy capsule in the infected animal, but there is little or no evidence of a capsule when the bacilli are cultivated on laboratory media. The pneumococcus, likewise heavily encapsulated in the infected animal, also forms a capsule in artificial media, but the capsule is best developed when the medium is enriched with blood or serum and carbohydrate. Among the pathogenic forms, capsule formation tends to diminish with continued cultivation on artificial media, probably because these furnish a somewhat less than optimal environment, and is restored by animal passage of the bacteria.

The heavily encapsulated saprophytic bacteria, such as the dextran- and levulan-forming cocci of the genus Leuconostoc, show no tendency to loss of capsule formation in culture provided that carbohydrate is supplied, and it seems probable that the artificial medium is more nearly optimal than it is in the case of the more fastidious pathogenic bacteria. In general, then, capsule formation is determined in large part by the environment, may be a quantitative matter and is reversible.

Capsule formation may be specifically inhibited, however, the most effec-

[2] Braun: Jour. Bact., 1946, 51:327.
[3] See Colwell: Jour. Bact., 1946, 52:417.

tive means being the inclusion of antiserum to the capsular material in the culture medium. The loss of capsule formation under such circumstances, or when it is relatively complete by thorough prolonged culture on artificial media, results in the S–R dissociation, and the lack of a capsule is characteristic of the rough variant. As indicated above, this loss is also reversible, though frequently only with considerable difficulty. Whether it is to be regarded as basically different from a gradual diminution in the amounts of capsular substance synthesized through continued cultivation in a less than optimal environment is not clear. The relatively rapid appearance of non-encapsulated variants may be a consequence of the highly selective effect of antiserum rather than indicative of a mutation-like change.

Flagella. The occurrence of flagella on motile bacteria is also subject to variation. There appears to be little or no variation with respect to numbers or position of flagella, but their presence or absence is variable. Non-motile variants of motile bacteria are occasionally, though not commonly, observed, and these appear to be stable forms with little tendency to reversion to the motile form. In the case of very actively motile bacteria such as Proteus, colonial morphology is affected by motility in that the motile bacteria "swarm" and the growth spreads in a thin film over the surface of an agar medium, while the non-motile variants form discrete colonies.

The formation of flagella is subject to environmental influence also. Actively motile species kept in stock culture on laboratory media tend to lose their motility, and potentially motile bacteria may be motile when cultivated at one temperature but not at another. The inclusion of antibacterial substances such as phenol in the medium in toxic but non-lethal concentrations inhibits the formation of flagella by bacilli of the Salmonella group; in fact, one method of obtaining these forms free from flagella and the antigenic substances associated with them is culture on nutrient agar containing 0.1 per cent phenol.

Spores. The spore is a consequence of the aggregation of cell substance within a spore case during spore formation. It is this process rather than the formation of a structure of the vegetative cell that is subject to variation. Like capsule formation it is essentially a physiological process but with morphological consequences. Spore formation is affected by environmental factors, and asporogenous variants which breed true also occur. In the case of the anthrax bacillus, for example, spores are formed only when the bacteria have access to free oxygen; only vegetative forms are found in the infected animal until the bacilli are exposed to air by tissue decomposition or autopsy and then spore formation occurs. Similarly, spores are formed most rapidly at 32° C., less so at 37° C., and not at all when the culture is incubated at 42° C. The inhibition of spore formation by the anthrax bacillus by high temperatures is a temporary one in that when the temperature of incubation is reduced spore formation again occurs, but if cultures are maintained at 42° C. for many transfers the ability to form spores is apparently permanently lost. In general, however, spore formation is a relatively stable character.

Involution Forms. The occurrence of aberrant or "abnormal" forms of bacteria is very common and observed most frequently in old cultures. As indicated earlier (p. 40), such forms are commonly regarded as involution

forms, that is to say, dead or dying cells, and it has been pointed out that the physical structure of dead bacteria breaks down, with the protoplasm becoming granular and escaping into the surrounding medium with disintegration of the cell wall (Fig. 5). In addition to such dead cells, filamentous and coccoid forms of bacilli, swollen cocci, and the like are found with some frequency.

The tendency to aberrant morphology is in part a function of the structural rigidity of the bacterial cell, and in part an effect of the environment on the cell. It is self-evident that this should be so in a macromolecular environment; for example, no small degree of structural rigidity is necessary to maintain a bacillary form in the face of the forces of surface tension, and any crumbling of that structure will of necessity result in misshapen cells.

Fig. 26. Involution forms of the typhoid bacillus; fuchsin stain. Note the filamentous forms and elongated rods mixed with the typical forms. What appear to be buds on the filament are probably adjacent cells. The poor resolution at these high magnifications is apparent. × 3500.

Thus, the non-sporulating anaerobic bacilli (p. 540) and the pleuropneumonia-like organisms (p. 547) are perhaps the most fragile of all bacteria, and in keeping with this have a highly variable morphology. Similarly, the cholera vibrio is a fragile bacterium, easily broken up by grinding procedures, and is notorious for its morphological instability.

In old cultures accumulated metabolic products not only affect the viability and reproduction of cells, but may well contribute more directly to the development of aberrant forms. Morphological variation may be produced experimentally by manipulation of the medium. If, for example, surface tension is lowered by the inclusion of a surface tension depressant, aberrant morphology results, and among the bacilli the rod form becomes elongated to give rise to filaments. Analogous effects are produced by the cultivation of bacteria in the presence of toxic but not lethal concentrations of salt. Another kind of effect is produced by the inclusion of antibiotic agents which inhibit cell division; the cells continue to metabolize but fail to divide, and assume swollen, misshapen forms.

It will be clear from the foregoing that a large proportion of morphologic-

ally abnormal bacterial cells are to be found in old cultures which arise as a consequence of the toxicity of accumulated metabolic products, and the disintegration of the structure of dead and dying cells. It is not so clear to what extent essentially normal physiological activity and ability to reproduce are associated with the structural integrity of the cell. Certainly some degree of distortion is not fatal; for example, the filamentous forms of bacilli produced by depression of surface tension of the medium are viable in many cases. It seems probable, however, that the physical organization of the cell is of no small importance, and in this connection it is significant that cultural studies of the aberrant forms found in old cultures have indicated that in the great majority of instances these forms are dead in that they are incapable of multiplication. A part, perhaps a very large part, then, of variation in the morphology of the bacterial cell is no more than the outward evidence of a dying population and the death and disintegration of individual cells.

Bacterial Life Cycles. Cyclical development of bacteria in a succession of morphological types is well known in some cases. The simplest is the spore-vegetative cell succession of the sporulating bacteria. The succession of morphological types, or cytomorphosis (p. 59), associated with the growth of the bacterial population discussed earlier, and the successive zoogleal and monad or swarmer stages of Nitrobacter also seem well established. Considerably more complex developmental cycles, or cyclogenies, analogous to those well known among the fungi and higher plants, have been postulated by some workers for many of the well-known bacteria such as the staphylococci, streptococci, enteric bacilli and the like.

The experimental evidence upon which the belief in bacterial life cycles is based consists of observations of aberrant, "abnormal" forms occurring in pure cultures. It is postulated that the so-called "normal" bacterial form is but one morphological stage of many in which a bacterium may exist but in which it is temporarily fixed by the constant environment of the laboratory medium. The appearance of aberrant forms is, then, evidence of a tendency on the part of the organism to assume the forms characterizing other stages of the life cycle, and such forms should not be termed involution or degenerative forms. The aberrant cells in turn break down to liberate minute viable organisms either directly or via a symplastic stage. The minute forms, designated as gonidia, gametes, microgametes and the like, may give rise to the "normal" vegetative cell, either directly or after a process of conjugation or sexual union.

In any attempt to weigh the evidence in favor of bacterial life cycles two points must be borne in mind. In the first place, the aberrant forms which constitute an essential part of the life cycle occur only in the death phase of a bacterial culture, never during the phases of active growth; and, in the second, many of the postulated forms are too small to permit resolution with visible light. Alternative explanations of aberrant morphology are many. Suppose the cell dies, autolysis follows with the breakdown of cell constituents to smaller molecules, the increase in the number of molecules in solution raises the osmotic pressure within the cell and the cell imbibes water with resultant distention and eventual disruption. Granular material may be volutin and similar granular substances commonly observed in bacterial cells.

It is perhaps significant that studies utilizing the micro motion picture tech-

nique for the continuous observation of the development of bacteria from a single cell have yielded no evidence of a bacterial life cycle.[4] The evidence for the existence of such life cycles is exceedingly tenuous for two reasons. Much of it is open to serious technical criticism,[5] and in all cases there are equally plausible alternative explanations. Aside, then, from the point of technical criticism, differences in opinion are largely a matter of interpretation and, in the absence of conclusive experimental evidence, which has as yet not been forthcoming, are likely to remain so for the time being.

Filterable Forms of Bacteria.[6] Perhaps associated with bacterial life cycles are the so-called filterable forms of these organisms. The visible, readily cultivable forms of bacteria such as are present in the ordinary "pure culture" do not usually pass through the finer-pored porcelain and infusorial earth filters. That there are particular conditions, however, that favor the transmissibility of certain bacteria through these filters has become increasingly evident. Aside from the technical difficulties that beset all filtration experiments there are serious divergences of interpretation. Many observers have reported that familiar microorganisms such as the tubercle bacillus and the typhoid bacillus can sometimes pass through well constructed filters and this they interpret as signifying the existence of a filterable stage in the life history of these bacteria. It is even alleged by some that the filterable viruses of smallpox, poliomyelitis and other diseases represent a filterable phase in the life cycle of visible microorganisms, streptococci or what not, a view that has been urged especially by Rosenow[7] in this country and Enderlein[8] in Europe. This view is by no means generally accepted.

On the other hand, it is maintained that the so-called "filterable" forms of the typhoid bacillus, for example, are merely cells dwarfed by inadequate nutrition or are viable cell particles so small as to pass through filters but with capacity of renewed growth when again placed in favorable surroundings. It may be urged that the ability of cell fragments to regenerate is no new thing in biology, and it is quite as plausible to regard minute filterable forms of cocci or tubercle bacilli as portions of fragmented cells as to look on them as representing a significant filterable phase in their life histories.

PHYSIOLOGICAL VARIATION

Variation in the physiological activities of bacteria is exceedingly common. It may take various forms such as alterations in virulence, *i.e.*, ability to produce disease, changes in fermentative activity and nutritive requirements, or the acquisition of resistance to antibacterial agents.

Attenuation. The term attenuation is ordinarily loosely used to mean reduced virulence of a pathogenic microorganism. It may indicate a simple loss of ability to produce disease in a general sense, or a reduction in virulence for one host species accompanied by an increase in virulence for another host species.

[4] Wyckoff: Jour. Exp. Med., 1934, 59:381.
[5] For example, see Holman and Carson: Jour. Inf. Dis., 1935, 56:165; also Lamanna: Jour. Bact., 1944, 47:327.
[6] Hadley, Delves and Klimak: Jour. Inf. Dis., 1931, 48:1.
[7] For example, see Rosenow: Amer. Jour. Clin. Path., 1944, 14:150.
[8] Enderlein: Arch. Entwicklungsgesch. Bakt., 1940, 1:252.

In general most pathogenic bacteria tend to lose virulence when kept in culture on artificial media. For example, pneumococci rapidly lose virulence for the mouse and in even a very few transfers on enriched media the minimal lethal dose increases by several hundred fold. Staphylococci, however, retain their virulence over many transplants on artificial media, but eventually become relatively avirulent. A few bacteria, such as the anthrax bacillus, seem to retain virulence almost indefinitely but this is not common. Such losses in virulence are general in character in that the bacteria become less virulent for a variety of experimental animals, and presumably arise as a consequence of adaptation to an environment other than that of the animal body. The artificial environment may be deliberately made somewhat toxic in order to bring about such changes. Simple aging in the case of Pasteur's cultures of the fowl cholera organism was sufficient to reduce virulence so that a fatal infection was not produced, and the attenuated strain of bovine tubercle bacillus known as BCG was carried on a bile-containing medium until its virulence was apparently completely lost.

Loss of virulence may be accompanied by changes in other characteristics such as marked diminution in capsule formation by the pneumococcus, a loss of golden pigment by *Staphylococcus aureus,* somewhat less fastidious nutritive requirements, etc. In many instances the change is dissociative in nature and loss in virulence is associated with a shift from the smooth form. In most bacteria, of course, virulence is markedly reduced by the S–R dissociation, and a change of this kind may occur *in vivo,* presumably under the influence of antibody; for example, the typhoid bacilli excreted by most chronic carriers are rough, avirulent forms.

Virulence may often be restored by animal passage, *e.g.,* successive and repeated infection and reisolation of the bacterium through a series of experimental animals. It cannot be concluded that the microorganisms are individually altered in the restoration of virulence by animal passage, and it is more probable that the animal acts as a highly selective screen, separating out those few bacteria in the inoculum which multiply most rapidly in the tissues. The restoration of virulence is usually accompanied by a restoration of other characters associated with it such as capsule formation.

The virulence of a microorganism may be modified by passage through another host species and sometimes, though not invariably, virulence for the original host species is reduced. The classic example of attenuation by animal passage is that of Pasteur's attenuation of the rabies virus by adaptation to the rabbit brain. The original strain from dogs, "street virus," kills rabbits in about two weeks following subdural inoculation, after some twenty passages it kills in eight days, and after an additional twenty or more passages the period may be reduced to seven days but cannot be reduced further. This "fixed virus" has acquired such a marked affinity for the nervous tissue that it will not produce rabies on subcutaneous inoculation and so may be used as an immunizing agent. Such attenuation or adaptation to a new host is very common among the viruses, and includes the adaptation of a variety of viruses to the chick embryo, the adaptation of yellow fever virus to the mouse brain, etc. Perhaps the best known single example is that of the conversion of smallpox virus to cowpox virus.

Attenuation of virulence is of considerable practical importance in relation to the preparation of immunizing agents. Among the bacterial diseases, killed vaccines are not effective immunizing agents in anthrax and tuberculosis, but attenuated anthrax bacilli were originally used by Pasteur to immunize sheep, and the attenuated strain of tubercle bacilli, BCG, has been of increasing interest as an immunizing agent in man. Among the virus diseases, effective immunity is rarely obtained with killed material, and vaccines of attenuated virus, often given in conjunction with antiserum, are very valuable.

Biochemical Variation. Variation in the biochemical properties of bacteria, such as fermentation of sugars, decomposition of proteins and amino acids, nutritional requirements, resistance to antibacterial agents, and the like occurs with some frequency. Changes may appear seemingly spontaneously, but more often seem to be in the nature of an adaptive response to the environment.

Fig. 27. Colonies of *Bact. coli mutabile* on lactose agar. Note the lactose-fermenting papillae of the variant appearing on the non-lactose-fermenting colonies (Parr).

Mutation-like Variation. The best known example of variation of this kind was first observed by Massini in 1907. He isolated a non-lactose-fermenting strain of *Bacterium coli* which, when cultivated on lactose agar containing an indicator which turned red in the presence of acid, gave rise to white colonies. On continued incubation, however, red papillae appeared on the colonies, indicating that some of the bacteria were decomposing the sugar to acids. This is illustrated in Fig. 27. Subcultures from the red papillae bred true and showed no tendency to revert to the non-lactose-fermenting form, while subcultures from the white portions of the colonies gave rise to white colonies on which red papillae appeared as before. He concluded that the change was a mutation and that some strains of the colon bacillus, which he named *Bacterium coli mutabile,* were genetically unstable with a tendency to throw off lactose-forming mutants. Such strains of colon bacilli have since been found repeatedly and the variation has been studied in some detail. It was found by Lewis[9] that if such strains were cultured on a synthetic medium containing lactose as the only source of organic carbon, a constant proportion,

[9] Lewis: Jour. Bact., 1934, 28:619.

about one in 100,000, of the cells were able to grow. Further studies by Deere[10] have shown that both fermenting and non-fermenting varieties contain the enzymes necessary for the lactose fermentation, and suggested that the fermenting variants differ from the parent strain in that the cell wall is permeable to lactose.

A similar mutation-like change with respect to citrate utilization by coliform bacteria has been observed by Parr and Simpson,[11] and extensive experiments with other members of the colon-typhoid-dysentery group of bacilli have shown that this kind of variation with respect to the fermentation of other substances such as dulcitol is not uncommon. A number of these organisms will, when cultivated on media containing a sugar they are unable to ferment, respond with the formation of such papillae, the tendency toward reversion to the parent type varying from one organism to another.

Mutation-like variation occurs in properties other than sugar fermentation. One of the most adequately studied of these is pigment production by *Bacterium prodigiosum*, which has been investigated by a number of workers, especially Bunting.[12] During growth of the culture white, pale pink, bright pink and dark red variants are thrown off at a relatively constant rate, one per 10,000 cells in the case of dark red and one per 3000 cells in that of bright pink, but, with rare exceptions in the case of white and pale pink, these variants do not breed true and on subculture show essentially the same color distribution as the parent strain.

Variation by Adaptation or Training. Changes in biochemical properties also occur in a seemingly more gradual way, induced by continued cultivation in appropriate media. Such variation in nutritive requirements is the basis for the general observation that the more fastidious pathogenic bacteria are frequently more difficult to cultivate on primary isolation and enriched media are required, while, after being carried in culture for some time, they grow more rapidly and profusely and on somewhat simpler media. For example, on primary isolation *Brucella abortus* requires an increased carbon dioxide tension, but after a few subcultures this may be dispensed with. Similarly, the gonococcus and meningococcus require, on primary isolation, both an increased carbon dioxide tension and an enriched medium, usually a heated blood medium. After a few subcultures, however, it is no longer necessary to supply carbon dioxide, and eventually growth occurs in simpler media.

This process of adaptation to growth on an originally deficient medium has been studied with respect to specific nutritional requirements. Fildes, Gladstone and Knight[13] found, for example, that some strains of the typhoid bacillus which require tryptophane could be trained to grow without this amino acid by successive culture in decreasing concentrations of it. Gladstone[14] similarly showed that some strains of *Staphylococcus aureus* that initially required a number of amino acids could eventually be adapted to grow in the presence of an ammonium salt as the only source of nitrogen, and

10 Deere: Jour. Bact., 1939, 37:355, 473.

11 Parr and Simpson: Jour. Bact., 1940, 40:467.

12 Bunting: Jour. Bact., 1940, 40:57, 69; *ibid.*, 1942, 43:585, 593; summarized Symp. Quant. Biol., 1946, 11:25.

13 Fildes, Gladstone and Knight: Brit. Jour. Exp. Path., 1933, 14:189.

14 Gladstone: Brit. Jour. Exp. Path., 1937, 18:322.

Koser and Wright[15] could adapt dysentery bacilli requiring nicotinic acid to growth in its absence. Ability to ferment carbohydrates may also be either acquired or markedly enhanced by continued cultivation in the presence of the substrate or by very heavy inoculation of highly specific media in which the substrate is the only nutritive material. Still other adaptations such as to changes in pH, increased incubation temperature and the like also occur, but are usually not marked.

Adaptive Enzymes.[16] The ability of a microorganism to decompose a substrate may be very rapidly enhanced in the presence of that substrate. Although known for many years, this particular kind of bacterial variation has been the subject of renewed interest as *enzyme adaptation*. The enzymes which may be formed under the influence of the presence of substrate have been called adaptive enzymes in contrast with those which are formed whether or not the substrate is present, the constitutive enzymes. The formation of adaptive enzymes may be demonstrated by cultivation of the bacteria in a medium containing the substrate and then testing the organisms for enzymatic activity, or a washed suspension of bacteria grown in the absence of the substrate may be mixed and incubated with it. In the first instance, activity is immediately apparent, the bacteria having formed the adaptive enzyme during growth, and in the second a latent period varying from thirty minutes to two to four hours occurs before rapid decomposition of the substrate begins.

Thus, washed suspensions of the colon bacillus bring about an equally rapid decomposition of glucose, as measured by oxygen uptake in respirometers, regardless of whether the medium upon which the organisms were grown contained this sugar. On the other hand, the enzyme system of this organism which is responsible for the decomposition of tryptophane to indol is an adaptive enzyme. If the medium upon which they are grown contained tryptophane, washed suspensions immediately convert the amino acid to indol, but if the medium did not contain tryptophane and the washed bacilli are suspended in tryptophane solution, decomposition to indol occurs only after a latent period. Similarly, the amino acid decarboxylases are formed for the most part in the presence of the substrates. Many of the bacterial proteases are adaptive enzymes, for only small amounts of the enzymes are formed in the absence of protein, but filtrates from cultures in protein-containing media are actively proteolytic. Various other bacterial enzymes are adaptive also; thus, the enzyme responsible for the reduction of tetrathionate to thiosulfate by *Salmonella paratyphi B*, tetrathionase, and that catalyzing the reduction of nitrate to nitrite by *Bact. coli*, nitratase, are adaptive.

The question of whether or not the formation of an adaptive enzyme is associated with bacterial multiplication is one of considerable interest. In general it appears that some degree of multiplication is essential (cell division occurs even in suspensions of washed bacteria), and it has been shown in the formation of galactozymase by *Bact. coli* that the amount of enzyme was proportional to the number of new cells.[17] In a few instances, however, adaptive

---

[15] Koser and Wright: Jour. Bact., 1943, 46:239.

[16] For general discussions see Karström: Ergeb. d. Enzymforsch., 1938, 7:350; Dubos: Bact. Rev., 1940, 4:1; Gale: Bact. Rev., 1943, 7:139.

[17] Stephenson and Gale: Biochem. Jour., 1937, 31:1311.

enzymes have been found to be formed by washed bacteria in the presence of the substrate and under circumstances in which there was no detectable cell division; these include the formic hydrogenylase of *Bact. coli*,[18] the galacto-zymase of *Saccharomyces cerevisiae*[19] and the nitratase and tetrathionase of *Salmonella paratyphi* B and *Bact. coli*.[20] The formation of adaptive enzymes by partially poisoned bacteria, *i.e.*, those which cannot reproduce but continue to metabolize, has, however, not been observed.

The distinction between adaptive and constitutive enzymes is not a sharp one, for frequently, as in the case of the bacterial proteases, the adaptive enzyme is not completely lacking in the absence of the substrate. It is clear that there is some relation between the formation of adaptive enzymes and adaptation to a substrate by serial culture in its presence in that the distinction appears to be in part a quantitative one. In bacterial growth enzyme adaptation is expressed in terms of the lag period and the generation time during the period of active growth. What might be called the population-growth expression of adaptive processes has been studied at some length by Hinshelwood and his colleagues.[21] They have shown that, in the adaptation of *Bact. aerogenes* to the most efficient utilization of substrates such as glycerol, disaccharides, glycine, etc., the adaptation is expressed as a transition from a slower to a more rapid rate of growth in the early stages as indicated by breaks in the growth curves, and as a decrease in mean generation time in the period of logarithmic growth, the latter decreasing from 70 to 110 minutes for unadapted strains to 32 to 33 minutes for adapted ones. The process of adaptation cannot be fully explained, however, on the basis of rate of formation of adaptive enzymes, for an adaptive enzyme, such as a bacterial protease, does not tend to persist as a constitutive enzyme when the bacterium is kept on protein-containing media for many transfers and then cultured in the absence of protein. An adapted strain tends to breed true to a certain extent in that there is a tendency for the acquired property to persist in the absence of the substrate, and the tendency is directly related to the extent to which adaptation is carried. Strains of bacteria vary, but in a general sense it might be said that following fifteen transfers in substrate-containing media, the property tends to disappear at about the same rate as that at which it was acquired, but if the process of adaptation has been carried on for, say thirty transfers, it may persist indefinitely in the absence of substrate.

Drug-Fastness. Microorganisms may become adapted to the action of antibacterial substances, and by a process of training acquire the ability to grow in concentrations that are bacteriostatic for the unadapted parent strain. This adaptation is of particular interest in relation to the antibacterial agents of chemotherapeutic importance, including certain of the dyes, the sulfonamide compounds, and the antibiotics, penicillin and streptomycin in particular. Such adaption occurs readily *in vitro* when the microorganisms are grown in increasing concentrations of the antibacterial agent, and there is convincing

---

[18] Stephenson and Stickland: Biochem. Jour., 1933, 27:1528.
[19] Stephenson and Yudkin: Biochem. Jour., 1936, 30:506.
[20] See Pollock: Brit. Jour. Exp. Path., 1946, 27:419.
[21] Hinshelwood: *Chemical Kinetics of the Bacterial Cell.* Clarendon Press, Oxford. 1946.

evidence that it also occurs *in vivo* though the relative importance of the latter phenomenon is not altogether clear. Strains of pathogenic microorganisms that have become resistant to the action of antibacterial substances in concentrations within a chemotherapeutic range are said to be drug-fast.

From a practical point of view it is significant that such adaptation can occur *in vivo*. Chemotherapy of the parasitic infections, such as trypanosomiasis, is of long standing and the acquisition of drug-fastness by such parasites is well known. Similarly, the development of strains of *Treponema pallidum* which are drug-fast to the arsenicals is observed from time to time. The occurrence of drug-fast strains of bacteria has assumed importance with the advent and general use of antibacterial agents effective in the chemotherapy of bacterial infections, first the sulfonamides and later the antibiotics. For example, a striking increase in the proportion of drug-fast gonococci occurred with the general application of sulfonamide therapy of gonorrhea. Carpenter *et al.*[22] observed an increase in the incidence of sulfonamide-fast strains of the gonococcus from 15 to 59 per cent in a period of fifteen months; similar results have been reported from Britain, and it is a common observation that at the present time the majority of cases of gonorrhea do not respond to sulfonamide therapy.

The prophylactic use of chemotherapeutic drugs also results in the development of drug-fast strains of bacteria. Thus, Siegal, Karr and Julianelle[23] found that sulfadiazine prophylaxis produced drug-fast pneumococci which spread, infecting some 90 per cent of the group under observation, and the institution of sulfadiazine prophylaxis of streptococcal infection in naval training camps in the United States during World War II was followed by a tremendous proportionate increase in drug-fast strains of streptococci within these groups which produced streptococcal infections that did not respond to sulfonamide therapy (p. 359). Cases of infection with sulfonamide-fast pneumococci have been found and the development of drug-fast strains during therapy and their spread to other persons have been observed; as yet, however, no widespread prevalence of infection with sulfonamide-fast pneumococci has been observed, and according to Hamburger *et al.*[24] the occurrence of drug-fast strains has not been quantitatively important in clinical practice as yet.

The widespread use of penicillin has resulted in an increase in penicillin-fast strains of staphylococci; in Hammersmith Hospital in London 14.1 per cent of strains isolated from lesions in the period April–November, 1946, were penicillin-fast, and this rose to 38 per cent for the period February–June, 1947.[25] Drug-fastness to streptomycin is especially prone to occur, and develops so rapidly in the infected individual undergoing therapy as to limit somewhat its chemotherapeutic value. It is not clear whether the observed increasing proportion of drug-fast strains of bacteria results from an adaptation to the drug *in vivo* and spread of the fast strain, or whether the drug-

[22] Carpenter, Ackerman, Winchester and Whittle: Amer. Jour. Pub. Health, 1944, 34:250.
[23] Siegal, Karr and Julianelle: Amer. Jour. Hyg., 1945, 41:228.
[24] Hamburger, *et al.*: Jour. Inf. Dis., 1943, 73:12.
[25] Barber: Brit. Med. Jour., 1947, ii:863.

fast strains exist prior to therapy and occur in larger proportion through elimination of susceptible strains by chemotherapy. It is not unlikely that both processes are operative, but their relative importance is not known. In any case it is clear that virulent, drug-fast strains of bacteria can and do develop and become disseminated through the host population.

As indicated elsewhere (p. 155), antibacterial agents affect the physiological processes of the cell, and it follows that the nature of the modification that results in drug-fastness is essentially one in the physiology of the bacterium. There is evidence for three general kinds of modification, *viz.*:

(1) If the drug acts by interference with some particular metabolic process, it is not unreasonable to suppose that the drug-fast strain may by-pass that process and make use of some alternative mechanism. If so it might be expected that the fast strain would differ physiologically from the parent strain. Such physiological differences have, in fact, been observed in sulfapyridine-fast pneumococci which, while dehydrogenating glucose at a rate equal to that of the parent strain, cannot dehydrogenate 3-carbon intermediates such as glycerol, lactate and pyruvate, while the parent strain can, and show also a marked diminution in hydrogen peroxide production.[26] If, however, the drug interferes with a synthetic process, the modification would not appear in the respiratory metabolism. While the processes of synthesis are much less well understood, there is evidence which suggests that alternative pathways of synthesis may be developed, as in the syntheses involving pantothenic acid catalysis by streptococci[27] and pantothenic acid and tryptophane metabolism of staphylococci.[28]

(2) Since the action of the sulfonamides is antagonized by *p*-aminobenzoic acid, it has been suggested that in some instances sulfonamide-fast strains of bacteria are resistant by virtue of an increased production of this or similar antagonists. In support of this it has been found that sulfonamide-fastness of staphylococci and gonococci is in some cases associated with the production of increased amounts of *p*-aminobenzoic acid,[29] and it has also been shown that pantolyltaurine-fast strains of the diphtheria bacillus are resistant because of their ability to synthesize pantothenic acid.[27] Specific antagonists of many antibacterial substances, including the antibiotics, are not known and the extent to which this general explanation of drug-fastness can be tested is not great as yet.

(3) A third possible explanation is that, if the drug acts as a competitive inhibitor of an essential metabolite because of similarity in molecular structure, the adaptation may consist in the development of the ability to metabolize the drug instead of the metabolite, giving rise to an apparently anomalous situation in which the essential metabolite shows antibacterial activity. A number of observations in support of this view have been reported. It was found by Wooley[30] that strains of the yeast *Endomyces vernalis* which were inhibited by pyrithiamine, an analogue of thiamine, could be made resistant

[26] MacLeod: Proc. Soc. Exp. Biol. Med., 1939, *41*:215.

[27] McIlwain: Brit. Jour. Exp. Path., 1943, *24*:203.

[28] Sevag and Green: Jour. Bact., 1944, *48*:615, 623, 631.

[29] Landy, Larkum, Oswald and Streightoff: Science, 1943, *97*:265; Spink: Jour. Exp. Med., 1944, *79*:331; Landy and Gerstung: Jour. Immunol., 1945, *51*:269.

[30] Wooley: Proc. Soc. Exp. Biol. Med., 1944, *55*:179.

by cultivation in its presence, and simultaneously acquired the ability to utilize pyrithiamine instead of thiamine. Emerson and Cushing[31] reported that a strain of the fungus Neurospora, made sulfonamide-fast by cultivation in the presence of sulfanilamide, not only utilized the drug but required it as an essential metabolite, and that the drug-fast strain was inhibited by *p*-aminobenzoic acid, the reverse of the behavior of the parent strain. Similarly, streptomycin-fast variants of meningococci were found by Miller and Bohnhoff,[32] some of which required streptomycin for growth, *i.e.*, were streptomycin-dependent, and others were only streptomycin-fast. Streptomycin-fast dependent variants of staphylococci, enteric bacilli, etc., have been described by Kushnik *et al.*[33] and by Paine and Finland.[34] The role of streptomycin as an essential metabolite for such variants is also strongly supported by the studies of Rake.[35]

There are, of course, still other explanations of the basis of adaptive resistance to antibacterial agents. For example, a degree of tolerance might also result from the development within the cell of substances which decrease the solubility of the drug and hence its ability to penetrate the cell; such an explanation has, in fact, been suggested to account for the tolerance which certain fungi may develop for disinfectants. Or the drug-fast variant may inactivate the antibacterial agent by decomposition; naturally occurring penicillin-resistant strains of staphylococci often produce penicillinase, and penicillin-inactivating substance is formed by some penicillin-fast variants.[36]

It is not necessary to assume that a single mechanism is operative in the development of drug-fastness, *i.e.*, those indicated above are in no sense mutually exclusive, and it is, in fact, probable that a variety are functional. For instance, while sulfonamide-fastness of gonococci is sometimes associated with an increased production of *p*-aminobenzoic acid as noted above, all sulfonamide-fast gonococci do not produce antagonists. Furthermore, more than one mechanism may be operative in a single adaptation; Davies and Hinshelwood[37] have reported, for example, that in the adaptation of *Bact. aerogenes* to sulfanilamide there is first a reduction in the prolonged lag period produced by the drug which they interpreted as the development of an alternative growth mechanism, and later an enhanced growth rate due to the formation of an antagonist.

The development of drug-fastness is most commonly a gradual process, though there are exceptions such as the streptomycin-fast dependent meningococci which appear on initial culture. The bacteria are usually cultured in increasing concentrations of the drug over an extended series of transplants and become more and more drug-fast in that successively higher concentrations are tolerated. These sometimes become almost fantastic in the light of the original sensitivity. The drug-fastness is specific also in that a sulfonamide-fast strain is normally susceptible to penicillin, etc. This specificity may

[31] Emerson and Cushing: Jour. Bact., 1947, 54:195.
[32] Miller and Bohnhoff: Jour. Bact., 1947, 54:467.
[33] Kushnik, Randles, Gray and Birkeland: Science, 1947, 106:587.
[34] Paine and Finland: Science, 1948, 107:143.
[35] Rake: Proc. Soc. Exp. Biol. Med., 1948, 67:249.
[36] See North and Christie: Med. Jour. Australia, 1946, 33:176.
[37] Davies and Hinshelwood: Trans. Faraday Soc., 1943, 39:431.

extend within a group of closely related compounds such as the sulfonamides, and Harris and Kohn[38] have reported that acquired resistance of *Bact. coli* to sulfanilamide did not always parallel that to sulfathiazole. Or it may extend to other compounds; thus Hinshelwood[21] has shown that *Bact. aerogenes* adapted to proflavine shows some "cross adaptation" to other acridines, methylene blue, and propamidine. In general, the specificity is greatest in the early phases of the adaptation. As indicated above, drug-fastness may be temporary in that reversion occurs on continued culture in the absence of the drug, but when the adaptation is carried over many transplants and to relatively high concentrations of the drug, it is apparently permanent. Reversion may be induced in some instances, however, by adaptation to some other drug, and acriflavine-fast *Bact. aerogenes* becomes susceptible to acriflavine when adapted to phenol.

Immunological Variation. The loss of capsules in the conversion of S to R forms is reflected, not only in the morphology and virulence of the organism, but also in its immunological character. The capsular material, usually polysaccharide in nature, confers a type specificity upon the organism, while the cell body proper contains antigens, designated as somatic, which confer an immunological relation upon closely related forms having similar or identical somatic antigens but different type-specific antigens. The change from S to R is, then, ordinarily accompanied by a loss of immunological specificity. The pneumococcus types, for example, differ from one another by virtue of type-specific capsular material and in the rough form lose their type specificity and become immunologically identical (p. 385).

Another type of colony variation or dissociation connected with the presence or absence of flagella was originally observed in cultures of *Proteus vulgaris*. The ordinary type of Proteus colony is irregular and tends to spread in a thin film over the surface of an agar medium because of the pronounced motility of these organisms, but occasionally compact, discrete colonies are observed. The spreading type has been designated by the letter H (German *Hauch* = film) and the discrete form by the letter O (German *ohne Hauch* = without film). The distinction between H and O forms has come to have considerable significance because of the difference in the antigens present in the flagella and those present in the cell body, the H or flagellar antigens conferring, like the capsular polysaccharide of some organisms, a type specificity upon the bacterium. The O antigens, though often referred to as somatic antigens, are by no means strictly analogous to the somatic antigens of the rough forms of bacteria, as will appear.

The nature of the H–O variation would appear to be different from that of the S–R variation in that the former takes place somewhat more readily and does not have a semipermanent character. The formation of flagellar antigen, for example, may be suppressed by cultivation of the bacteria on phenol agar, a suppression which is immediate but not permanent, for transfer to nutrient agar results in the prompt reappearance of the H antigens. Furthermore, the H antigens of Salmonella have been found to be of two kinds, specific and non-specific (the non-specific having even broader group affiliations than the O antigens). Bacterial species having both specific and

[38] Harris and Kohn: Jour. Immunol., 1943, 46:189.

non-specific H antigens are designated as diphasic. When plated out they give rise to approximately equal numbers of morphologically identical but immunologically distinct types, one having specific H antigens and the other non-specific H antigens. This variation would appear to be of a fluctuating type, since the two types of colonies breed true for but a brief time, both reverting after one or two transfers to mixtures of the two types. (See also p. 438.)

The relations between the H–O and the S–R types of variation are not as yet clearly understood. In general, as has been stated, the R types are non-motile, but motile (MR) strains sometimes occur, and, conversely, non-motile S types (NMS) are observed. The conversion of some of the Salmonella species from the S to the R form is accompanied not by a loss of flagellar antigens but by an alteration in the immunological specificity of the somatic antigens, possibly attributable to the loss of polysaccharide.

The Transmutation of Immunologic Types. Perhaps one of the most important contributions of recent years to the problems of immunologic variation among the bacteria is the alteration of the pneumococcus types. It was shown by Griffith that if a Type 1 pneumococcus, for example, is made to lose its type-specific polysaccharide (capsule) by conversion to the R form, then injected into an animal together with a suspension of heat-killed smooth Type 2 pneumococci, the living organism cultivable from the animal is a Type 2. It was later found that this transmutation of type could be brought about in the test tube by cultivation of the R form in the presence of anti-R immune serum and heat-killed smooth pneumococci of another type. This remarkable change is well established, and errors of experimental technique have been eliminated. Furthermore, Avery and his co-workers have isolated a substance from Type 3 pneumococci, consisting principally, if not solely, of a highly polymerized viscous form of desoxyribonucleic acid, which in minute amounts will induce the transformation of R variants of Type 2 to smooth, encapsulated, virulent Type 3 cells.[39]

A similar type transformation of colon bacilli dependent upon the presence of a capsule has been reported by Boivin and his co-workers.[40] Bruner and Edwards[41] were unable to bring about analogous changes in Salmonella but could induce changes in the O antigen within O subgroups (p. 439) by cultivation in the presence of absorbed O antisera. Profound changes in the H antigenic complex, including the development of unknown phases of monophasic Salmonella, may be produced with some facility by cultivation in the presence of appropriate antisera.[42]

Another instance of the acquiring of a new immunologic character by the typhoid and paratyphoid A bacilli has been reported by Holtman.[43] These organisms could be made to acquire the ability to form heterophile antigen (p. 278) by growing them in the presence of this substance in laboratory media or by growth in a collodion sac in the peritoneal cavity of the guinea

[39] See the review by McCarty: Bact. Rev., 1946, *10*:63.
[40] Boivin *et al.*: Experimentia, 1945, *1*:334; Compt. Rend. Soc. Biol., 1945, *221*:646, 718; *ibid.*, 1946, 222:1357.
[41] Bruner and Edwards: Jour. Bact., 1948, *55*:449.
[42] See the summary by Edwards and Moran: Proc. Soc. Exp. Biol. Med., 1946, *61*:242.
[43] Holtman: Jour. Immunol., 1939, 36:405, 413.

pig. In contrast to the artificial pneumococcus types, the acquired ability was not permanent but was lost after twenty to fifty transfers on ordinary laboratory media. In this connection it is of interest to recall that many years ago Smith and Reagh[44] suggested that bacteria might be immunologically altered by residence in different hosts. They were unable to demonstrate such differences, however, and accounted for the failure by the high degree of adaptation to the host of the forms studied.

MECHANISMS OF VARIATION

The picture presented by a consideration of the several kinds of bacterial variation appears to be somewhat confused. This is attributable in part to the nature of the characters subject to variation and the lack of their counterparts in the conventional genetics of higher organisms, and in part to the seeming lack of relation of the bacterial variations to one another, making systematization difficult. The confusion is more apparent than real, however, for the development of physiological genetics, and especially studies on fungi such as yeasts and Neurospora, have made it clear that physiological and immunological characters are subject to genetic control and may be regarded as quite as substantial as the morphological characters of conventional genetics. Furthermore, while perhaps overtly dissimilar, the kinds of bacterial variation may be in large part equated on a physiological basis. In the past few years great advances have been made in the study of bacterial variation and, while information is as yet fragmentary and there is no general agreement on many points, the underlying patterns are beginning to emerge.

Mutation.[45] The term mutation has come to be used loosely in bacteriological literature to mean any discontinuous variation. Such a definition is inaccurate in that a discontinuous variation may not result from a mutation in the conventional sense, and an apparently slow adaptive modification may have a genetic basis.

The stability of bacteria is of a very high order; the Rawlings strain of the typhoid bacillus, for example, has been maintained in culture for more than forty years, and still remains physiologically and immunologically identical with freshly isolated strains over what has been some thousands of generations. Such stability is indicative of an effective mechanism controlling the formation of enzymes, which give a bacterium its biochemical character, and the synthesis of antigenic cell substance. Thus there is a heredity-controlling mechanism which is presumably not dissimilar to that of higher organisms and subject to alteration or mutation. It is not material whether or not such a mechanism is differentiated as chromatinic bodies or similar structures.

Definitive evidence that mutation is responsible for a given variation is almost always lacking, but there seems to be no doubt that mutations do occur. The evidence which establishes this is the effect of irradiation in stimulating the production of variants, and the demonstration of recombination of biochemical characters occurring in the irradiation-induced variants. As pointed

[44] Smith and Reagh: Studies from the Rockefeller Inst., 1904, *1*:270.
[45] Summaries and discussions by various workers may be found in *Heredity and Variation in Microorganisms,* Cold Spring Harbor Symposia on Quantitative Biology, *11*, 1946; also see Luria: Bact. Rev., 1947, *11*:1.

out elsewhere (p. 143), the effect of ionizing radiation is a local one, confined to zones of intense ionization, and when irradiation gives rise to variants it would seem clear that a discrete directive mechanism has been affected. This is substantiated by recombination, presumably through a process of conjugation, with the production of hybrid types. This has been studied in detail by Lederberg[46] who has been able to define the position of the determinative units with relation to one another, *i.e.*, map the structure of bacterial chromosomes which necessarily remain hypothetical in the present state of uncertainty regarding the differentiation and structure of the bacterial nucleus.

Such definitive evidence as that of recombination is limited in application and as yet is available only in studies of variants of a single bacterial strain. Other kinds of evidence, however, are consistent with the assumption of mutation as a cause of bacterial variation. Thus irradiation-induced mutation is not qualitatively different from, but a stimulation of, spontaneous variation, and similar stimulation has been observed on treatment with nitrogen mustards (β-chloroethylamines) though not with colchicine. Quantitative studies have shown that the probability of occurrence of a variety of kinds of variants is 10^{-5} to 10^{-10} per bacterium per generation, one consistent with mutation rates observed in higher organisms, and may be raised to as much as 10^{-2} by irradiation. Furthermore, since the spontaneous rate is very low but when variation occurs it breeds true (otherwise it would not be detectable), the number of variants found in each of a series of cultures of not too large volume fluctuates erratically. This "fluctuation test" has been applied by Luria[45] and a number of other workers as an indicator of the occurrence of mutation. In addition, the occurrence of variants is independent of the environment though a selective environment may be required for the demonstration of their presence, *e.g.*, drug-fast variants. Generally speaking, the characters in which variation or mutation occurs are independent, such as specific growth requirements, but in some instances a variant arises through a series of step-like mutations, and in others there is some evidence of linkage. The application of one or more of these criteria has, in the great majority of studies, been the basis of judgment as to the function of mutation in the observed variation.

A large part of bacterial variation can be accounted for on the basis of mutation and selection for the phenotype so produced. Thus, as Braun[47] has shown, the S–R dissociation can be interpreted as the result of spontaneous mutation, or perhaps a series of mutations, in which the R form arises from the parent S form, persisting and eventually displacing it through the combined effect of a greater vitality and growth rate. The reversibility of the dissociative change may be attributed to reverse mutation; the R form appears to be stable because of its competitive advantages, but if the environment is altered to favor the S form as by the inclusion of anti-R serum, the S form again becomes evident. Small colony variants are regarded as mutants with a low metabolic rate.

Similarly adaptive responses such as attenuation in virulence, variation in nutritive requirements and sugar fermentations, and the development of drug-fastness are expressions of selection through an altered environment of spontaneous mutations which occur continuously and which, because of the rapid

46 Lederberg: Genetics, 1947, *32*:505.
47 Braun: Bact. Rev., 1947, *11*:75.

rate of cell division and the occurrence of bacteria as large populations, give an appearance of plasticity in adaptation to the environment. For example, in adaptation by training in which an essential metabolite is continuously reduced in successive cultures, the bacteria requiring the metabolite are put at increasingly greater disadvantage and a mutant which is able to synthesize the metabolite outgrows it. In the same way the cultivation of *Bact. coli mutabile* in lactose-containing liquid media will give the appearance of an adaptive response to the presence of lactose, with the culture becoming a more active fermenter with successive transplants, but this is no more than a selection of the lactose-fermenting variant by a favorable environment. Other processes in which varying degrees of adaptation are demonstrable, as in the development of resistance to antibacterial agents or drug-fastness, may be regarded as a consequence of a series of successive, step-like mutations and have been so interpreted. Certain of the immunological variations may likewise be interpreted as mutations; the loss of ability to synthesize specific polysaccharide results in loss of specificity in capsular or O antigens, and diphasic Salmonella may be regarded as forms in which there is a high mutation and reverse mutation rate in the mechanisms determining the synthesis of the flagellar antigens.

While mutation has been clearly demonstrated to occur in the bacteria, and undoubtedly underlies much of the observed bacterial variation, the above represents an extreme view in that some of the phenomena are susceptible of other interpretations supported by equally sound evidence, as will appear. In addition, some kinds of bacterial variation cannot be accounted for as a selection of spontaneously arising mutants. The kind of morphologic variation giving rise to involution forms is, of course, an obvious consequence of the physicochemical environment, but the purely physiological variations of transmutation of immunologic character, and the formation of adaptive enzymes in the absence of cell division, are not susceptible to explanation on the basis of mutation.

Enzyme Balance. Mutation resulting in alteration of nutritive requirements and sugar fermentations, and the development of resistance to antibacterial agents, necessitates not only the constant occurrence of a large number of mutations, but also that the mutations be such that the physiology of the bacterium is adjusted to the new environment with a very high degree of precision. Such a situation would appear somewhat improbable. If, however, the kind and proportion of enzymes present within the cell are affected by metabolism of the substrate, the adaptation can consist of an adjustment of the cellular physiology to utilization of that substrate with maximum efficiency. This is, in effect, a generalization of the phenomenon of enzyme adaptation, and has been most fully and convincingly developed by Hinshelwood.[48]

The physiological processes of the actively growing cell may be regarded as a series of interrelated reactions in which the product of one is the substrate of another, as in the catalysis of respiration. Or the linkage may be cyclical, one of the later reactions in the sequence providing an intermediate functioning in one of the earlier reactions in conjunction with some other substrate, as in

[48] Hinshelwood: *The Chemical Kinetics of the Bacterial Cell*. Clarendon Press, Oxford. 1946.

the cyclical processes of carbohydrate metabolism like the alcoholic fermentation, the Krebs cycle, etc. The individual reactions are determined by the concentration of intermediates, the amount of enzyme and the reaction velocity. A steady state occurs during logarithmic growth, but with the exhaustion of food materials, accumulation of metabolic products and the like, there is cessation of the maximum growth rate and concentrations of diffusible intermediates fall, and the individual enzymes decay and alter in their relative proportions as the system moves toward a new equilibrium. On transfer to fresh medium, readjustment is initiated during the lag period with the building up of concentrations of intermediates and a synthesis of enzymes in a shift toward an equilibrium consistent with maximum growth rate. Thus, the relative proportions of the elements of the catalytic system and the concentrations of substrates of the individual reactions exist in a series of metastable states of enzyme balance under ordinary culture conditions. For example, the formation of bacterial deaminases is suppressed during rapid growth in the presence of fermentable carbohydrate and does not occur at acid reactions but decarboxylases are formed at an acid reaction, and both kinds of enzymes are formed in the later stages of culture growth; the kind and proportion of enzymes present is a function of the age and condition of the culture. Similarly, the products of carbohydrate metabolism depend upon environmental factors, again a matter of balance in the function of the cellular enzymes.

If a strain of bacteria is transplanted to a medium differing from the one in which it has been grown, a greater degree of adjustment occurs. For instance, *Bact. aerogenes* ferments both glucose and lactose, but if cultured continuously on glucose and then transferred to lactose, the growth rate, measured as the mean generation time, is reduced. After several transfers in lactose the rate increases and the strain grows as rapidly in lactose as it formerly did in sucrose. If the training to lactose is only partial, reversion occurs in non-lactose-containing media, but if the strain has been carried through many transplants in lactose, the ability to use the sugar with a maximum efficiency persists. Since the bacterium ferments both sugars with facility the adaptation is not overt, but it is, nevertheless, a typical adaptation, and the result is clearly a consequence of alteration in the balance of enzymes already existing in the cell. The adaptive process is much more striking, and less obviously a result of change in enzyme balance, when there is a great differential between the parent and adapted strains, as between no detectable acidity and an active fermentation, whether it occurs slowly on successive transfer or very rapidly as in the formation of an adaptive enzyme.

The change in enzyme balance may be quantitative in nature. The enzyme in question may be present only as a precursor, the formation of enzyme occurring in the presence of substrate through mass action as suggested by Yudkin[49] for adaptive enzyme formation. Or the enzyme may be initially present in only very small amount; on the basis of comparison between number of molecules of certain vitamins per cell and the turnover rate of several enzymes, McIlwain[50] indicates how some enzymes may be present as only one or a few molecules. Or, finally, catalysis of the new reaction may be a function of an

[49] Yudkin: Biol. Rev., 1938, *13*:93.
[50] McIlwain: Nature, 1946, *158*:898.

existing enzyme but at a relatively low reaction velocity, and adaptation a matter of expansion of that enzyme. The last opens the question of qualitative modification of the enzyme, in that distortion of its specificity by a slightly different substrate requires greater activation energy and hence a lowered reaction rate, but if the substrate is present during formation of the enzyme, and the distortion not too great, a modified pattern may eventually result. This is consistent with much of the observed data but there is no direct evidence regarding it.

Drug-fastness is also satisfactorily explained in terms of enzyme balance. As indicated earlier, drug-fastness may result from the production of an inhibitor such as *p*-aminobenzoic acid in excess amounts, a qualitative modification such that the drug displaces the antagonist as an essential metabolite, or by diversion to an alternate metabolic pathway. The first of these is clearly an expansion of an existing catalytic system. Regarding the second, suppose it be assumed that the drug displaces the prosthetic group of an enzyme, *viz.*, panto- lyltaurine, to give a modified enzyme and the formation of different metabolic products. The adaptation can thus consist of an altered enzyme balance to allow the metabolism of the new intermediates, and when this is established, the original essential metabolite competitively inhibits the new system and the drug has become an essential metabolite. The development of an alternate metabolic pathway is likewise an expansion of an existing enzyme or system of enzymes which ordinarily contributes in but small amount to the main- tenance of the concentration of a given intermediate, perhaps because of lower reaction velocity. When the function of the usual system is inhibited by the drug, the alternate is expanded, perhaps only quantitatively, to neutralize the effects of the lower reaction velocity. The whole is analogous to an industrial system in which a raw material for a given process is shut off, an alternative, more expensive process is developed and the economies effected in large scale operation make it as efficient as the original process.

THE INTERPRETATION OF THE PHENOMENA OF VARIATION

The significance of the phenomena of bacterial variation to biology is un- certain. Because of unique environmental factors interpretation of the morpho- logical variation of individual cells presents unusual difficulties, and neither the mechanisms underlying this type of variability nor its relation to variation in anatomical structures of higher organisms is apparent as yet. Variation in colonial morphology, however, appears to be associated, in part, with physio- logical and immunological variation, and it is not unlikely that changes in col- ony form arise as a consequence of alterations in the nature of the cell surface.

The significance of the apparent loss and gain of physiological characters, even if these be absolute in that a property is *completely* lost or absent, is difficult to assess in general biological terms. As Needham[51] has pointed out, although a structure which makes possible a given physiological function may not be regained after being lost, the organism may re-acquire the function through the development of another and different structural mechanism. It is clearly open to question as to whether an enzyme system lost or gained by a

[51] Needham: *Contributions of Chemical Physiology to the Problem of Reversibility in Evolution.* Biol. Rev., 1938, 13:225.

8

bacterium may be regarded in the same category as an anatomical structure such as a tooth.

The immunological characters of bacteria, however, have their counterparts among the higher organisms in which immunological characters are known to be inherited, as in the case of human blood groups, or parallel phylogenetic relationships of accepted zoological classifications, as in the immunological relationship of blood proteins. In this connection it has been shown[52] that the immunological character of serum proteins of pigeons is genetically determined. If immunological characters of bacteria are to be regarded as fundamentally significant, it appears that on the one hand bacteria are unstable in this respect, viz., the constant fluctuation of specific and non-specific flagellar antigen in the diphasic Salmonella, and on the other, that such characters may be acquired through environmental influence. The fluctuating variation between specific and non-specific H antigens and the random redistribution of these antigens in the bacterial population following selection may well indicate, however, that some, at least, of these immunological characters may not be of fundamental biological importance, a suggestion that is, in part, supported by present knowledge of common antigens and partial antigens.

This apparent acquisition of new immunological characters is also of interest in a somewhat different connection, that of the apparent autocatalytic properties of some substances. Recent studies have indicated that some of the filterable viruses are proteins. Since these agents are able to increase in the body of the host, it would appear that they stimulate the host cells to form more virus protein. The analogy to the transmutation of pneumococcus types and the acquiring of the heterophile antigen by the typhoid and paratyphoid A bacilli is obvious.

Bacterial Phylogeny. From the evidence of comparative bacterial physiology (Chapter 4) it appears not unlikely that bacteria were among the first living organisms on the earth. The autotrophic bacteria may plausibly be regarded as primitive forms of life that could exist in an environment containing no organic matter, and that have persisted until the present time. Teleologically, it would seem that in the bacteria nature tried a variety of energy-yielding mechanisms ranging from the oxidation of inorganic compounds of nitrogen, carbon, sulfur, iron, manganese, etc., to the utilization of radiant energy by the photosynthetic forms. The last process, however, became of quantitative importance in the green plants rather than in the bacteria. With the accumulation of organic matter, transitional forms, persisting today as the facultative autotrophes, arose in which there appeared mechanisms for the oxidation of organic compounds; concurrent with this development came the ability to respire in the absence of molecular oxygen. The wide variety of bacteria which exist at the present time may well be regarded as a consequence of the physiological expansion made possible by the accumulation of a variety of organic compounds and the development of respiratory mechanisms that made possible their utilization. In this connection it is of interest that vestiges of autotrophic physiology, such as the ability to oxidize hydrogen, persist in some of the heterotrophic bacteria.

With this development of respiratory mechanisms, however, a series of de-

[52] Cumley, Irwin and Cole: Proc. Nat. Acad. Sci., 1941, 27:565.

generative changes appeared, the first being the loss of the ability to reduce carbon dioxide, followed by an increasing dependence upon preformed organic compounds as building stones for protoplasm and upon preformed components of the enzyme systems concerned in respiration, such as coenzyme, thiamine and the like. Degeneration appears most marked in the pathogenic forms (possibly reaching an ultimate in the filterable viruses if, as some think, these are microorganisms completely dependent upon the host cells for enzyme systems), and it is possible that this degeneration has been accelerated through parasitism.

Such an approach necessitates the assumption that the autotrophes appeared fully developed and in very many respects this seems highly improbable. An alternative which avoids this difficulty is the assumption that the formation of organic matter, through the condensation of small polyfunctional molecules with aggregation into macromolecules, preceded the appearance of living cells, and that these were essentially heterotrophic. From such heterotrophic ancestors the autotrophic bacteria could develop as the supply of preformed organic matter ran low, and present heterotrophic forms utilizing organic matter of green plant origin differentiated either from or coincidentally with the autotrophes.

Parasitism.[53] Although the ability of some bacteria to produce disease may be a purely fortuitous coincidence, *viz.*, the tetanus bacillus, many of the pathogenic forms have, through long association, become adapted to life in the body of the host to such a degree that they are unable to survive in nature or possibly even on artificial culture media or in the bodies of animals closely related to the particular host. This seems, for example, to be the case with the leprosy bacillus, which, so far as is known, is not able to grow anywhere except in the body of man and possibly of the anthropoid ape. Theobald Smith has suggested that bacteria of great pathogenic power should be regarded as incompletely adapted parasites that have not yet succeeded in establishing an equilibrium between themselves and their host. The less complete the adaptation, the more virulent the disease produced. This concept would explain the tendency of long established diseases to decrease in severity at the same time that they are becoming more prevalent.

This adaptation to a parasitic mode of existence is indicated in a variety of ways. The nutritive requirements of some of the pathogenic bacteria, for example, such as the necessity for fresh blood, ascitic fluid and the like in laboratory media, suggest an adaptation to an environment in which these or similar substances are available—the tissues of the host. The optimum temperatures for the pathogenic bacteria are, without exception, the body temperatures of their host; the human type of tubercle bacillus grows best at 37° C. but the avian type is presumably adapted to the higher body temperature of the bird, 41° to 42° C.

The question of whether a host may, through long association, become adapted to a parasitic bacterium to such a degree that the presence of the microorganism is either necessary or of advantage to the continued existence of the host is an open one. In certain cases the presence of a parasitic bacterium

[53] *Cf.* Smith, Theobald: *Parasitism and Disease.* Princeton University Press, Princeton. 1934.

bears a significant relation to the assimilation of food by the host; such, for example, is the case with respect to the leguminous plants and the root nodule bacteria, and the herbivorous animals and cellulose-decomposing microorganisms. Whether the abundant intestinal flora of man and other animals in general functions in a similar fashion has been the theme of considerable speculation and some investigation. Nuttall and Thierfelder early showed that guinea pigs removed by cesarean section and kept in a sterile environment survived for ten days or so, but Schottelius was not successful in rearing bacteriologically sterile chicks. Nutritional inadequacies of the chick diet apparently accounted for these results, for it was later shown that chicks could be raised for as long as forty days in a bacteria-free environment. The work of Reyniers[54] and his colleagues in recent years has shown that not only chickens, but a variety of higher animals such as guinea pigs, rabbits, rats, etc., may develop in the complete absence of bacteria. It appears likely, therefore, that an intestinal flora is not essential to the continued existence of some of the higher animals, but whether it may be an advantage is as yet uncertain.

[54] Reyniers: *Micrurgical and Germ-Free Techniques: Their Application to Experimental Biology and Medicine.* Charles C Thomas, Springfield, Ill. 1943.

THE CLASSIFICATION OF BACTERIA

The classification of bacteria presents peculiar difficulties that stem more or less directly from their simplicity of structure. Since taxonomy in general rests on a morphological or anatomical basis, the relationship, phylogenetic or otherwise, of bacteria to the higher forms is difficult to define, and the interrelations of the bacteria themselves present problems to which taxonomy has, to date, offered no entirely satisfactory solution. A consequence of this structural simplicity is the widespread use of physiological characters in the differentiation of the bacteria into genera and species, characters whose counterparts among the higher organisms are, in general, regarded as of but minor taxonomic importance. As has been pointed out previously, differences that are quite possibly trivial biologically are often of great practical importance and hence have come to assume a taxonomic significance that may often be entirely undeserved. In other cases the biological importance of a given characteristic or group of characteristics is unknown, and the inability to assess the significance of characters proposed as differentials has led to disagreement among bacteriologists.

Such a situation clearly does not lend itself to order and system nor, and perhaps most important of all, does it allow the development of a classification which is based upon characters of fundamental significance and which shows the biological or genetic relationship of these organisms to one another. The practical consequences are two: there is no classification of bacteria which is generally accepted in more than its primary subdivisions; and, second, the bases upon which various groups of bacteria are classified differ widely, as will appear, from one group of organisms to another. It should be emphasized, therefore, that bacterial "species" are by no means analogous to the species of the zoologist or botanist and cannot be compared directly to them.

The phenomenon of bacterial variation, discussed in the previous chapter, is clearly of no small importance in the classification of these organisms. From the general biological point of view there is but little solid ground upon which a sound taxonomic structure can be built. In the practical identification of bacteria, however, such variations are not a source of great embarrassment. As has been pointed out, the majority of bacterial variations arise as a consequence of alterations in the environment and, in a working sense, may be regarded as adaptations. The observed uniformity of the morphology and biochemical properties of the bacterial culture is due to the fact that observations of these characters are always made in the same way. It is only when the procedure is varied, either in terms of the past history of the bacterial culture or in the

way in which the test is conducted, that variability becomes apparent and the illusory nature of the supposed constancy of these organisms is demonstrated. The question of whether bacteria should be regarded as members of the plant or animal kingdoms is no longer considered of great importance. Possessing characteristics of both, these organisms may be regarded as a connecting link between the plants and animals. Using the term bacteria in its broad sense, the microorganisms included under this head make up a continuous series of types which appear to connect the two kingdoms. The so-called higher bacteria, the sheathed, filamentous forms, are closely akin to the fungi; bacteria such as the tubercle bacillus and the diphtheria bacillus appear to be further removed from the plants but show some fungoid characters, such as tendencies to branching and filament formation. Among the well-known forms such as the streptococci, staphylococci, sporulating rods and gram-negative intestinal forms, the morphological relations to the fungi are less apparent; organs of locomotion appear, and with the spiral forms the bacteria merge into the protozoa. Physiological characters and the evidence of chemical composition of the cells tend to reinforce this intermediate position. The metabolism of these organisms is chemosynthetic, an animal-like character, but the ability to utilize ammonium salts as a source of nitrogen suggests a plant type of physiology; nucleic acids found only in plant cells or only in animal cells have all been found in the bacteria. It was proposed many years ago that a third kingdom be created for these organisms, the *Protista,* but since the dividing lines between such a third group and the plants on the one hand and the animals on the other are as vague as that between the plants and animals, the proposed new kingdom would solve no difficulties and has never been accepted. It is generally agreed, however, that the sum total of bacterial characters allies them more closely to the plants than to the animals, and they are classified with the plants as *Schizomycetes* or fission fungi (German *Spaltpilzen*). The relationship of the Schizomycetes to other plants may be indicated as follows:

Phylum   I. *Thallophyta,* plants without distinction of root, stem and branch
              Subphylum 1—the algae
              Subphylum 2—the fungi—thallophytes lacking chlorophyll
                  Class   I. *Schizomycetes*—the bacteria
                  Class   II. *Myxomycetes*—the slime molds
                  Class III. *Phycomycetes*—the algae-like fungi
                  Class IV. *Ascomycetes*—the fungi forming ascospores
                  Class   V. *Basidiomycetes*—the fungi forming basidiospores
Phylum   II. *Bryophyta*—the mosses
Phylum III. *Pteridophyta*—the ferns
Phylum IV. *Spermatophyta*—the seed-bearing plants

Although the interrelationships of the organisms included under Schizomycetes present problems which are, in many essentials, new to the taxonomist, certain "natural" groups are apparent. Of these the most obvious is that based on morphology, with primary division into spherical, rod-shaped, spiral and filamentous forms, and subdivision on the basis of spore formation, presence and location of flagella, and staining reactions to the gram and acid-fast stains. The earlier classifications, the better known of which are those of Migula and of Lehmann and Neumann, were made on this basis. These classifications

exerted a strong influence on bacterial taxonomy and resulted in the naming of a great many species of bacteria, many of which persist in current classifications.

Morphology is, however, not a sufficient basis for the separation of bacterial species, for many morphologically similar organisms may be quite different in other respects. Physiological differences, generally readily determinable in the laboratory, have been widely used. In fact, one of the early classifications, that of Orla-Jensen, depended upon nutritional differences for the primary subdivision into three main groups:

1. The autotrophic bacteria which, like the green plants, require neither organic carbon nor organic nitrogen compounds

2. Bacteria which require organic carbon compounds but can dispense with organic nitrogen, using ammonium salts or other inorganic compounds of nitrogen

3. Bacteria which, like the higher animals, require both organic carbon and organic nitrogen compounds

A number of workers, especially Kluyver and van Niel,[1] are of the opinion that comparative physiology should constitute the primary basis of separation, rather than being subordinate to morphology.

Beyond such primary subdivisions, classification becomes increasingly difficult and it is quite clear that this is so because not nearly enough is known of the phylogenetic relationships of the bacteria to one another for a detailed classification with definition of genera and species. This question has been discussed in some detail by van Niel[2] who shows that as yet any classification can be little more than a key and species only "form" species, that is to say, no more than convenient handles.

Bacterial classification has evolved through some sixty odd schemes to perhaps half a dozen current today.[3] The criteria made use of are, in the order of approximate fineness of distinction made:

(1) morphology—both gross and microscopic,
(2) physiology—biochemical properties,
(3) pathogenicity—of the disease-producing bacteria,
(4) immunology—the antigenic structure of the bacterial cell.

Of the current classifications that of the Bergey Manual[4] is by far the most detailed and is the only one that need be considered here. It developed from the earlier classification of Chester and the work of a committee of the Society of American Bacteriologists, but is not, as is sometimes supposed, the official expression of the views of the Society. The Bergey classification has gained ascendancy in this country in recent years, though it is seldom used outside the United States.

An abridged outline of the Bergey (1948) classification is given in the accompanying diagrammatic form. It differs from the Bergey system in that the genus Staphylococcus is retained, and the tribe Hemophilae is not broken

---

[1] Kluyver and van Niel: Centralbl. f. Bakt., Abt. II, 1936, *94*:369.
[2] van Niel: Symp. Quant. Biol., 1946, *11*:285.
[3] For a discussion of bacterial classification and its development see Buchanan: *General Systematic Bacteriology*. Williams & Wilkins Company, Baltimore. 1925.
[4] Bergey: *Manual of Determinative Bacteriology*. 6th ed. Williams & Wilkins Company, Baltimore. 1948.

Order	Family	Tribe
		Nitrobacterieae....................
	Nitrobacteraceae.............	Hydrogenomonadeae..............
		Thiobacilleae.....................
	Pseudomonadaceae..	Pseudomonadeae..................
		Spirilleae..........................
	Azotobacteraceae....................................	
	Rhizobaceae...	
	Micrococcaceae......................................	
	Neisseraceae..	
	Lactobacillaceae.	Streptococceae.....................
		Lactobacilleae.....................
Eubacteriales (suborder Eubacterineae)	Corynebacteraceae.	
	Achromobacteraceae..................................	
	Enterobacteraceae.............	Eschericheae......................
		Erwineae.........................
		Serrateae.........................
		Proteae...........................
		Salmonelleae......................
	Parvobacteraceae..............	Pasteurelleae......................
		Brucelleae.........................
		Bacteroideae......................
		Hemophileae ....
	Bacteriaceae..	
	Bacillaceae...	
Actinomycetales.........Actinomycetaceae		
Chlamydobacteriales..		
Myxobacteriales...		
Spirochaetales.........	Spirochaetaceae.....................................	
	Treponemataceae....................................	

194

SLIGHTLY MODIFIED FROM BERGEY (1948)

Genus

⎡Nitrosomonas......... ⎤
⎢Nitrosococcus......... ⎬ the nitrifying bacteria (p. 78)
⎣Nitrobacter........... ⎦

..Hydrogenomonas........the hydrogen bacteria (p. 83)

..Thiobacillus.............certain of the sulfur bacteria (p. 80)

⎡Pseudomonas...........Ps. pyocyaneus et al. (p. 529)
⎢Methanomonas.........methane bacteria
⎣Acetobacter.............acetic acid bacteria

⎡Vibrio.................cholera, paracholera and non-cholera vibrios (p. 478)
⎢Cellvibrio..............cellulose oxidizing vibrios (p. 86)
⎢Thiospira...............sulfur vibrios
⎣Spirillum...............Sp. minus (rat-bite fever) and saprophytic species (p. 752)

..Azotobacter............non-symbiotic nitrogen-fixing bacteria (p. 111)

⎡Rhizobium..............symbiotic nitrogen-fixing bacteria (p. 113)
⎢Agrobacterium..........plant pathogens and saprophytes
⎣Chromobacterium.......certain pigmented saprophytic bacteria

⎡Staphylococcus..........the staphylococci (p. 346)
⎢Gaffkya................M. tetragenus and related bacteria (p. 354)
⎣Sarcina.................S. lutea and other cocci

⎡Neisseria..............gonococcus, meningococcus, etc. (p. 395)
⎣Veillonella..............certain anaerobic cocci (p. 413)

⎡Diplococcus............pneumococcus and related forms (p. 381)
⎢Streptococcus...........the streptococci (p. 355)
⎣Leuconostoc............saprophytic cocci

⎡Lactobacillus.......... ⎫
⎢Microbacterium....... ⎬ lactic acid bacteria (p. 531)
⎣Propionibacterium.......propionic acid bacteria (p. 537)

⎡Corynebacterium........diphtheria and diphtheroid bacilli (p. 606)
⎢Listeria.................L. monocytogenes (p. 538)
⎣Erysipelothrix..........bacterium of erysipeloid and swine erysipelas (p. 673)

⎡Alcaligenes..............Alc. fecalis and related forms (p. 463)
⎢Achromobacter..........non-pigmented soil and water bacteria
⎣Flavobacterium.........pigmented soil and water bacteria

⎡Escherichia........... ⎫ coliform bacteria (p. 418)
⎢Aerobacter........... ⎬
⎣Klebsiella..............Friedländer's bacillus (p. 425)

..Erwinia................plant pathogens

..Serratia ....Bact. prodigiosum and related forms

..Proteus................Pr. vulgaris and related forms (p. 428)

⎡Salmonella.............typhoid and paratyphoid bacilli (pp. 432, 448)
⎣Shigella................dysentery bacilli (p. 464)

⎡Pasteurella.............plague and hemorrhagic septicemia bacilli (p. 502)
⎢Malleomyces...........glanders bacillus (p. 599)
⎣Actinobacillus..........actinobacillosis (p. 672)

..Brucella...............bacilli of undulant fever and contagious abortion (p. 492)
..Bacteroides.............non-sporulating obligate anaerobes (p. 540)
..Hemophilus............influenza bacillus (p. 515), pertussis bacillus (p. 520), chancroid bacilli (p. 526)

..Bacterium.............miscellaneous non-spore-forming bacilli of uncertain status

⎡Bacillus................aerobic, spore-forming bacilli such as B. anthracis, B. subtilis, etc. (p. 556)
⎣Clostridium............obligate anaerobic, spore-forming bacilli, including bacilli of tetanus (p. 568), gaseous gangrene (p. 575) and botulinus bacillus (p. 591)

⎡Nocardia...............aerobic, sometimes acid-fast actinomycetes (p. 591)
⎣Actinomyces...........anaerobic actinomyces of actinomycosis (p. 659)

.......................filamentous saprophytic bacteria, including sheathed iron bacteria (p. 82)
.......................slime-forming saprophytic bacteria having a pseudoplasmodial stage

⎡Spirochaeta
⎢Saprispira   ....saprophytic water forms
⎣Cristipira..............parasite of molluscs

⎡Borrelia................relapsing fever spirochetes (p. 727)
⎢Treponema............spirochetes of syphilis (p. 735) and yaws (p. 743)
⎣Leptospira.............spirochetes of infectious jaundice and field fever (p. 746)

down into genera other than Hemophilus. These and minor variations which appear later have been retained to keep the nomenclature from differing too widely from practice in other than current American literature. For example, the name *Vibrio comma* for the cholera vibrio is not used outside the Bergey classification; over 98 per cent of the papers published on this bacterium in the past 20 years are not American, and consistently use the name *Vibrio cholerae*. Abridged keys for the differentiation of the better known species of bacteria are included in subsequent chapters.

Nomenclature.[5] Throughout the development of bacteriology names have been given these organisms in a haphazard fashion and often with singular disregard for the conventions of botanical nomenclature. In general, however, a bacterium has a generic name, always written with a capital letter, which may or may not be descriptive (for example, *Bacillus*—a small rod; or *Pasteurella*—in honor of Pasteur) and a specific name which may be an adjective (*albus*—white) or a noun indicating possession (*Clostridium welchii*—Welch's clostridium) or a noun in apposition (*Bacillus radicicola*—the root-dweller bacillus). The practice of indicating the author of the name, as in *Bacillus subtilis* Cohn, is not as common in bacteriology as in zoology or botany. The use of trinomial and quadrinomial names such as *Granulobacillus saccharobutyricus mobilis nonliquefaciens* is obviously highly undesirable. Trinomials are occasionally useful in the designation of varieties of subspecies. Common names are, of course, used; terms such as Friedländer's bacillus, the typhoid bacillus, etc., are frequently encountered.

Genera. Perhaps one of the greatest difficulties the bacterial taxonomist labors under is the paucity of genera. The question of what degree of difference shall be judged sufficient to establish new genera is one to which there is as yet no satisfactory answer. At the present time considerable confusion and lack of uniformity still exist. In a number of instances new generic names have obtained wide currency among bacteriologists in many parts of the world, partly, doubtless, because they are applied to fairly distinct groups of microorganisms and have genuine classificatory value. Such are *Brucella* (for the bacilli of undulant fever of man and contagious abortion of cattle), *Salmonella* (for the paratyphoid bacilli) and *Pasteurella* (the bacilli of hemorrhagic septicemia in domestic animals and the bacilli of plague and tularemia); the name *Shigella* for the dysentery bacilli has gained a considerable degree of recognition. Without any formal international agreement, these names can be said to have won international standing.

In a number of instances it seems necessary to mention both old and new names whenever a microorganism is referred to. Few bacteriologists venture to use the name *Serratia marcescens* without explaining that they mean *Bacterium prodigiosum* or *Bacillus prodigiosus*, or *Gaffkya tetragena* without the equivalent *Micrococcus tetragenus*. Similarly the adherence of bacteriologists to various taxonomic schemes gives rise to a series of names for a microorganism; the familiar typhoid bacillus may be *Bacterium typhosum*, *Bacillus typhosus*, *Salmonella typhi*, *Eberthella typhosa* or *Salmonella typhosa*.

For the present the bacteriologist seems to have no escape from using a

[5] Rules of nomenclature, proposed but not officially adopted, are discussed by Buchanan, St. John-Brooks and Breed: Jour. Bact., 1948, 55:287.

double set of generic names for certain organisms, or from using the older, albeit nomenclatorially unjustifiable, names for others, while welcoming certain new and useful generic designations such as Brucella and Salmonella. Uniformity, consistency and strict adherence to the rules of biological nomenclatorial practice are perhaps in the future.

Species. The differentiation of species is made for the most part on a physiological basis. A single character is hardly sufficient, particularly in view of the facility with which variation occurs. The use of a number of such characters is the rule, but difficulties are frequently encountered when intermediate forms occur. For example, *Bact. coli* and *Bact. aerogenes* are almost universally regarded as different species and in the Bergey classification are put into different genera, Escherichia and Aerobacter respectively. Yet forms intermediate between the two are so commonly encountered that it sometimes seems a doubtful procedure to attempt to separate these so-called coliform bacteria.

Types. Immunological differentiation is generally regarded as not of species status though it may sometimes coincide with species differentiation on a physiological basis. Conversely, minor cross reactions between species are not indicative of species identity. There are some exceptions to the former, however, and many workers divide the genus Salmonella into species on the basis of antigenic structure. Immunological differences are of very considerable value, particularly for epidemiological purposes, and are usually the basis of distinction of types within a species. Thus the streptococci are divided into groups, designated A, B, C, etc., on the basis of one kind of antigen, and into arabic numbered types within and across species by another. The pneumococci are similarly divided into numbered types on the basis of capsular antigen. *Clostridium botulinum* is separated into types, A, B, C, etc., on the basis of the immunological specificity of the toxin, and these have no relation to the immunological character of the cell substance. There is, then, no general practice with respect to type differentiation. Physiological differences within species are usually not used to differentiate types, and the biochemical reaction is simply given as variable.

Chapter 8

THE RELATION OF BACTERIA TO DISEASE

Prior to the discovery of the causal relation of bacteria to the infectious diseases, these afflictions were regarded as an outward manifestation of the activity of some metaphysical or supernatural agency. A common belief, and one that still persists among some primitive peoples, was that of demoniacal possession, the invasion of the human body by the demon, taking the form of punishment for misdeeds or a consequence of individual failure to take adequate precautions against malignant spirits. When the advance of knowledge brought a larger measure of understanding of the structure and functions of the human body, a new and semiscientific theory of disease sprang into being and, although not entirely displacing the concept of demoniacal invasion, attained world-wide influence. The Hippocratic theory of disease, as it was called after its founder, the Greek physician Hippocrates, postulated four bodily humors, blood, phlegm, yellow bile and black bile. Health consisted of a proper mixture of these humors, disease an improper mixture. Although supplanted to a mild degree in the seventeenth century by more complex and mystical theories such as the homeopathy of Hahnemann, the Hippocratic doctrine of humors was dominant throughout the middle ages and even yet colors much medical thought and practice.

Amid the vagueness and confusion of these half mystical hypotheses emerged the tangible and definite germ theory of disease. As already pointed out, the germ theory of disease is the legitimate offspring of the germ theory of fermentation, and owes its origin to the memorable investigations of Louis Pasteur. The belief that the infectious diseases are caused not by demons or improper mixtures of humors or by any spiritual dynamic derangement but by microscopic plants and animals is now securely established on a firm experimental foundation.

Koch's Postulates. The unequivocal proof of a suspected causal relation between a given bacterium and a particular disease is dependent upon the development of a logical chain of experimental evidence which is often formalized as a series of postulates. These are commonly known as Koch's postulates although there is no evidence that Koch himself expressed these experimental steps in terms of formal "postulates." There are four of these:

(1) The bacterium must be observed in every case of the disease.
(2) The bacterium must be isolated and grown in pure culture.
(3) The bacterium, in pure culture, must, when inoculated into a susceptible animal, give rise to the disease.
(4) The bacterium must be observed in and recovered from the experimentally diseased animal.

Clearly, if each of these steps can be carried out, the evidence implicating the bacterium as the causative agent of the disease is very strong indeed.

Although in a great many cases this chain of experimental evidence can be developed, or, as sometimes said, Koch's postulates can be fulfilled, and the story is in its essentials complete, in a considerable number of diseases one or more of these steps cannot be carried out. In this connection, these experimental steps are best considered one by one.

The *first postulate* may be fulfilled in practically all cases if the term "observe" may be taken in its broad sense. Bacteria, of course, can be observed in the literal sense, but the submicroscopic filterable viruses cannot be seen with the aid of ordinary equipment. Their presence may be demonstrated, however, by animal inoculation, and it is not unreasonable to regard such a procedure as observation in a very real sense. It should be pointed out here that in the early days of bacteriology it was assumed that the bacterium under suspicion should be present only in cases of disease and not in healthy individuals. It is now well known that the corollary does not necessarily follow. A number of pathogenic bacteria, such as those causing diphtheria, typhoid fever and the like, may be present in a virulent form in healthy persons who show no clinical symptoms.

The *second postulate* is, in general, somewhat more difficult to satisfy, although in most cases a causative bacterium may be isolated and grown in pure culture. Perhaps the best known example of an organism that has not been cultivated outside the body of the host is the leprosy bacillus (see, however, p. 647), and as a consequence it cannot be said with certainty that this bacterium is responsible for the disease. The ultramicroscopic viruses have not been cultivated on lifeless media and, although these agents may be shown to proliferate in tissue culture or in the developing chick embryo (p. 850), there is no positive assurance that such cultures are "pure," *i.e.*, that a virus is but a single entity and not a mixture of two or more viruses.

The *third postulate* is, in practice, probably the most difficult one to fulfill. The disease must be reproduced in a clinically recognizable form; a localized infection or a general septicemia is not sufficient although often suggestive. Since the most important diseases, from the anthropocentric point of view, are the diseases of human beings, it is necessary to find an experimental animal that will respond to the infection in the same or nearly the same manner as the human subject. A satisfactory experimental animal which complies with this requirement may be difficult to find, for phylogenetic relations are not necessarily correlated with susceptibility; monkeys, chimpanzees and the like are not, in general, better experimental animals than rabbits, guinea pigs, etc. The importance of the experimental animals to the study of an infectious disease is obvious; epidemic influenza, for example, in spite of the tremendous amount of concerted study which followed the 1918 epidemic, was but poorly understood until it was discovered (1933) that the disease could be reproduced in the ferret. The difficulties sometimes encountered in the experimental reproduction of a disease very likely stem in part from the adaptation of the bacterium to a parasitic mode of existence in conjunction with a particular host species.

The *fourth postulate* generally offers no difficulties if the preceding three

can be satisfied. Its importance should, however, not be minimized, for the presence of the microorganism in the experimentally infected animal is indicative of its proliferation and invasion of the host tissues.

A fifth postulate is sometimes added to the original four, namely: the injection of the products of a bacterium should give rise to the clinical symptoms of the disease. This postulate is applicable to those organisms which form soluble or exotoxins, such as the diphtheria and tetanus bacilli, but it is not of general significance in the incrimination of a bacterium as a cause of disease.

As indicated above, not infrequently one or more of these postulates cannot, or has not, been satisfied with respect to a given disease. The question then arises as to whether the suspected microorganism may be held responsible for the disease on the basis of only partial compliance with Koch's postulates. There is, unfortunately, no general answer to this; each case must be considered on its own merits. In some diseases, such as leprosy, the actual evidence consists in the observation of an association of a particular bacterium with a certain clinical syndrome and pathology. Whether the association may be interpreted in terms of causality is, of course, quite uncertain and consequently the etiology of leprosy is, as noted above, not definitely known. The absence of other possible etiologic agents may be regarded as favorable, though weak, ancillary evidence, and *Mycobacterium leprae* is generally, though tentatively, considered to be the cause of this disease.

In other cases indirect evidence assumes a more important position; in that of the virus diseases, for example, while there is no absolute assurance that a tissue culture represents a pure culture of virus, the homogeneity of the infective agent is rendered highly probable by facts such as the immunological relation existing among strains of a virus (*e.g.*, influenza or poliomyelitis) and the relative constancy of the epidemiology and pathology of the disease. The inability to isolate a filterable virus in "pure culture," then, is not a serious deterrent to the implication of these agents in the causation of disease.

The case of typhoid fever is somewhat different, for here, although the organism may be grown in pure culture, the clinical picture of the disease cannot be reproduced in the laboratory animal. Indirect evidence such as the epidemiology of the disease, the undoubted efficacy of immunization with killed suspensions of the typhoid bacillus in pure culture, etc., is so strong that the disease may be regarded as almost certainly caused by this microorganism, even in the absence of the inadvertent laboratory infections of human beings which have supplied the missing link in the chain of evidence.

It is clear, then, that while the fulfillment of Koch's postulates is, in general, essential to the proof of the bacterial etiology of a disease, in some instances in which a part of the necessary direct evidence is lacking the weight of ancillary evidence may be sufficient to make the postulated causal relation highly probable or, occasionally, practically certain. It may be noted at this point that the logical processes contained in Koch's postulates are by no means confined to the study of infectious disease; the responsibility for a given fermentation, for nitrogen fixation, etc., is fixed upon a particular microorganism by essentially the same logical development of experimental evidence.

Even though a bacterium has been shown to be pathogenic, *i.e.*, capable of producing disease, the outcome of a chance contact between such a micro-

organism and a prospective host is variable. The result of this contact may be negative with no clinically apparent departure from the normal physiological state regarded as health, or obvious disease of varying degree of severity may ensue. The nature of the outcome is dependent upon the interaction of two factors, the virulence of the organism and the resistance of the host. Both virulence and resistance are vague terms which express a combined effect of a variety of component factors. Some of these are known in each case, although the manner in which they function is generally not well understood, and there are undoubtedly many more which are unknown and possibly unsuspected at the present time.

VIRULENCE

The precise meaning of the term virulence is difficult to define in an entirely satisfactory fashion. Some workers include the ability to form soluble toxins as a part of the virulence of a bacterium; toxigenic diphtheria bacilli, for example, are said to be "virulent" while the atoxigenic diphtheroids are regarded as "avirulent." Others assume virulence to be synonymous with invasiveness, the ability of a microorganism to invade the tissues of the host. Under the circumstances it is perhaps best to disregard these more subtle distinctions and to define virulence as the ability of a bacterium to produce disease, thereby making virulence and pathogenicity synonymous.

The Measurement of Virulence. By definition virulence can be measured only in terms of the production of disease. In man it is usually judged on the basis of case fatality rates, a highly virulent microorganism producing death in a large proportion of the infected persons, and one of low virulence only a small number of deaths. Similarly, the titration of virulence (or any lethal agent such as a toxin) in the experimental animal is based on deaths, but dosage, which is inversely related to virulence, can be varied. Thus virulence (or toxicity) may be defined as the dose which will produce the specified deaths in a specified animal.

In the earlier studies virulence was measured as the minimum lethal dose, defined as that amount of the substance to be tested which would just kill the experimental animal, and definitions of toxin units, etc., are given in terms of these limits (p. 288). In recent years it has become generally apparent, however, that an accurate measure is essentially statistical in nature. It is assumed, and not unreasonably, that the resistance of the normal animal to the lethal agent rests on a probability basis and is normally distributed, *i.e.*, is described by the Gaussian frequency distribution. If this function is integrated, an S-shaped curve is given by the plot of the integral which describes accumulated deaths plotted against dosage. The point of inflection is, of course, that dose with which 50 per cent of the animals die. This is called the *median lethal dose* or LD_{50} dose. This dose can be calculated by a relatively simple procedure[1] from the accumulated deaths from graded doses extending through the 50 per cent end point.

For purposes of comparison between, for example, normal and treated animals, determination of the LD_{50} doses allows a relatively precise measure having a standard basis. A somewhat less satisfactory but comparable pro-

[1] Reed and Muench: Amer. Jour. Hyg., 1938, 27:493.

cedure is that of relative survival times. A variety of other methods of analysis may be used for purely comparative purposes. For example, control and experimental death rates may be tested for statistical significance by the point binomial or fourfold table. Comparative death ratios, such as 8/10 to indicate that 8 animals died of 10 inoculated, are almost useless from the quantitative point of view.

Toxins. It is difficult if not impossible to account for the derangement of the normal physiological processes and attendant clinical symptoms of an animal suffering from an infectious disease on the basis of a purely mechanical effect of the presence of the invading bacteria in the blood or other tissues. It was early discovered that a number of the pathogenic bacteria produce poisonous substances which, when absorbed by the host, give rise to the symptoms and pathology characteristic of the particular disease. It has been pointed out previously (p. 124) that these poisonous substances or toxins fall into two categories: (a) the *soluble toxins, exotoxins* or *true toxins* which apparently diffuse out of the intact bacterial cell into the surrounding culture medium or tissue; and (b) the *endotoxins* which do not diffuse out of the intact bacterial cell but may be separated from it *in vitro* by mechanical disruption of the cell structure. These substances are liberated *in vivo* through destruction of the bacterial cells by the various defensive mechanisms of the host.

The soluble toxins, although not a part of the cell substance, cannot be regarded as secretions in the usual sense of the word. The rate of appearance of toxin does not, except in the case of *Clostridium welchii,* parallel the rate of growth of the culture; toxin is liberated in the greatest amounts after the phase of active growth is over and many of the cells are dead or dying. The endotoxins appear to be a part of the cell substance of bacteria containing them; organisms such as the cholera vibrio, certain of the dysentery bacilli and the like may be regarded as microorganisms whose protoplasm is toxic to the higher animals which they infect. There are a number of differences between these two types of toxins which may be summarized as follows:

Exotoxins	Endotoxins
(1) Occur outside the bacterial cell.	(1) Intracellular in the intact cell.
(2) Poisons of extremely high potency.	(2) Low potency.
(3) Excellent antigens which give antisera of higher titer.	(3) Poor antigens, antisera of low potency.
(4) Combine with antibody according to the law of multiple proportions.	(4) Do not combine in multiple proportions.
(5) Protein in nature.	(5) Some are proteins but some are glucolipoids.
(6) Thermolabile.	(6) Thermostable.
(7) Destroyed by proteolytic enzymes.	(7) Resistant to the action of proteolytic enzymes.
(8) Detoxified by formaldehyde.	(8) Toxicity not affected by formaldehyde.

These differences are by no means absolute, and the listing of them represents a generalization to which there are a number of exceptions, as will appear. Consequently, the identification of a toxin as an exo- or endotoxin can be made, not on the basis of a single property, but only on the aggregate of the properties it exhibits. Even when all its properties are taken into consideration, a given

toxin, such as the Shiga dysentery bacillus toxin, for example, may occupy an anomalous position.

The soluble bacterial toxins are, by a comfortable margin, the most potent poisons known; only the vegetable poisons, ricin and abrin, approach them in potency. The most potent of the soluble toxins are those formed by *Clostridium botulinum, Clostridium tetani* and the diphtheria bacillus. Crystalline type A botulinum toxin (p. 593) is the most potent of the bacterial toxins; the LD_{50} for the mouse is only 4.5×10^{-9} mg. N. Type B botulinum toxin is somewhat less potent, 5 to 9×10^{-9} mg. N/LD_{50} for the mouse, and crystalline tetanus toxin 5 to 7.5×10^{-7} mg. N/LD_{50}. Highly purified but non-crystalline preparations of diphtheria toxin kill guinea pigs in amounts of 0.4 μg. per kilo body weight. While these amounts seem fantastically small—for example the LD_{50} of crystalline type A botulinum toxin is the equivalent of 2.1×10^7 molecules—the effective concentration in the body is much higher in that these toxins have marked affinities for certain tissues, as that of botulinum and tetanus toxins for the nervous tissue. Other toxins, such as that of *Clostridium welchii*, are much less potent, and it may require as much as 0.1 ml. of culture filtrate to kill an experimental animal. Still less potent are the endotoxins whose minimum lethal dose may be as much as one million times greater than that of a potent exotoxin. The MLD of a killed broth culture of the cholera vibrio, for example, is about 0.5 ml. for the guinea pig.

The potency of these toxins is, in general, paralleled by their efficiency as antigens. Antitoxic sera of high titer, 1 ml. of which will neutralize thousands of guinea pig MLD's, may be obtained against diphtheria toxin, but antitoxic sera prepared against endotoxins are generally of very low titer—several milliliters may be required to neutralize 1 to 5 MLD's of endotoxin. The neutralization of exotoxin by antitoxin proceeds according to the law of multiple proportions, *i.e.*, if x units of antitoxin neutralize y units of toxin, nx units of antitoxin will neutralize ny units of toxin (see p. 288). The neutralization of endotoxin does not proceed in this orderly manner; it may be possible to protect an animal against 4 to 5 MLD's by the injection of antiserum, but if much more endotoxin is given, greater amounts of antiserum will not provide protection.

The nature of toxins, the soluble toxins in particular, has been a subject of long-continued interest.[2] The soluble toxins appear to be proteins; they are denatured by heat, may be salted out of solution, etc. Highly purified diphtheria toxin has been prepared by Eaton[3] and by Pappenheimer[4] and probably represents the pure toxin; the preparations appear to be homogeneous protein. Type A toxin of *Clostridium botulinum* has been prepared in crystalline form by Lamanna, McElroy and Eklund[5] and by Abrams, Kegeles and Hottle.[6] The former used an initial acid precipitation followed by shaking with chloroform and salting out with ammonium sulfate, and the latter a combination of sodium sulfate and acid precipitation. Both preparations crystallized in the form of

[2] See the review by Eaton: Bact. Rev., 1938, 2:3.
[3] Eaton: Jour. Bact., 1936, 31:347.
[4] Pappenheimer: Jour. Biol. Chem., 1937, 120:543.
[5] Lamanna, McElroy and Eklund: Science, 1946, 103:613.
[6] Abrams, Kegeles and Hottle: Jour. Biol. Chem., 1946, 164:63.

needles and were pure protein, having the properties of globulin and a molecular weight of about 1×10^6. Type B botulinum toxin has been prepared as a pure homogeneous protein, though not crystallized, by Lamanna and Glassman.[7] Tetanus toxin has been prepared in crystalline form by Pillemer, Wittler and Grossberg[8] by methanol precipitation in the cold and similarly appears to be pure protein. These results definitely establish the protein nature of at least these bacterial exotoxins.

With the exception of botulinum toxin, the soluble toxins are destroyed by proteolytic enzymes; botulinum toxin is, therefore, the only toxin which is effective when given by mouth. There is no chemical evidence which explains, even in part, the toxicity of these substances; these proteins do not seem to differ in any essential respect from bland proteins, such as egg albumin. The amino acid composition of crystalline type A botulinum toxin, for example, is in no way unusual. It has been suspected that toxicity might be a property of a prosthetic group attached to the protein molecule, but this appears not to be true; the balance of evidence indicates that the toxicity is a property of the structure of the toxin molecule, possibly of the arrangement of the constituent amino acid molecules in the protein.

Evidence as to the mode of action of the bacterial exotoxins is, however, beginning to accumulate. The α toxin of *Clostridium welchii* is a lecithinase and the enzymatic activity accounts in large part for the hemolytic and other toxic properties of this substance. The Welch bacillus also produces a collagenase or κ toxin which, by attacking muscle collagen, may be at least partially responsible for the pulping of muscle seen in human gangrene. Pappenheimer[9] has reported evidence which strongly suggests that diphtheria toxin may represent the protein moiety of an iron-containing respiratory enzyme, formed in abundance when the bacilli are grown in the presence of minimal amounts of iron; possibly its toxicity may be due to a competitive inhibition of respiratory enzymes of the host (p. 153). The mode of action of neurotoxins such as tetanus toxin is not yet understood.

The discovery that the toxic qualities of the soluble toxins are destroyed by treatment with formaldehyde which, at the same time, leaves the antigenic and antitoxin-combining properties of the toxin unimpaired, has been of the greatest practical importance in immunization procedures. Toxin so treated is called *toxoid* or *anatoxin*. It might be supposed that this observation would throw some light on the nature of the toxicity of these substances since formaldehyde is known to block amino groups—a fact taken advantage of in Sørensen's formal titration of amino acids. Analysis of the formaldehyde-protein derivatives indicates that the aldehyde combines with primary amino and primary amide groups, but not with secondary amide or phenolic groups. Because of the effect of formaldehyde on toxicity and the results of detoxification with ketene and phenyl isocyanate, some workers have supposed that the free amino groups of the toxin molecule, such as the ϵ-amino group of lysine, are intimately associated with toxicity, but the evidence for this is by no means complete.

[7] Lamanna and Glassman: Jour. Bact., 1947, 54:575.
[8] Pillemer, Wittler and Grossberg: Science, 1946, 103:615.
[9] Pappenheimer: Jour. Biol. Chem., 1947, 167:251.

The endotoxins are, in general, much more resistant to heat and to the proteolytic enzymes than are the soluble toxins. Many require heating to 80°–100° C. for one hour, and an endotoxin isolated from the meningococcus requires one hour at 120° C. for destruction; in contrast, the most resistant of the soluble toxins, botulinum toxin, is destroyed by exposure to 80° C. for ten minutes. Some of the endotoxins are destroyed by the proteolytic enzymes, but others appear to be immune to the action of these ferments. Similarly, formaldehyde has little or no effect on the majority of the endotoxins.

Some of the endotoxins may be protein, and it is thought by some that these substances are not toxic *per se* but are broken down in the animal body, either in the cells or body fluids, to toxic split products. The endotoxins of the enteric bacilli have been investigated in some detail and have been found to be poly-saccharide-lipid-polypeptide complexes. The complex is broken down by mild acid hydrolysis and the toxicity disappears, though in some instances the lipid fraction may retain a small degree of toxicity. There is also some evidence that the endotoxin of the cholera vibrio is closely associated with a phospholipid fraction of the cell. Substances such as these would not, of course, be affected by formaldehyde or proteolytic enzymes.

Following the injection of exo- or endotoxins, a period of incubation elapses before symptoms of intoxication appear. This incubation period may be very short or may extend to thirty-six or forty-eight hours or longer. The usual incubation period of tetanus toxin is thirty-six hours but it may be reduced to thirty-five to sixty minutes by the injection of very large amounts, 500,000 MLD, of crystalline toxin. It is sometimes stated that endotoxins have no incubation period, thereby differing from the exotoxins, but this is not generally true.

The pharmacological action of the soluble toxins resembles that of the vegetable alkaloids and is sometimes quite definite and characteristic for each toxin. Diphtheria toxin, for example, produces degeneration of the heart muscles, kidneys and liver and the hemorrhagic reaction in the adrenals which is a highly characteristic postmortem finding in the guinea pig. A part of tetanus toxin (tetanospasmin) has an affinity for the motor nerves. On the other hand, the symptoms and pathology resulting from administration of the endotoxins are not at all characteristic although minor distinctions have been reported. Animals inoculated with lethal doses of these substances usually show dyspnea, diarrhea and, in some cases, flaccid paralysis of the posterior extremities, become progressively weaker and finally die without having exhibited symptoms that might be regarded as characteristic of a particular endotoxin. The histopathology often indicates that the toxin exerts its effect on the blood vessels, damage to which results in degenerative changes in the tissue supplied. It has also been found[10] that these substances affect the carbohydrate metabolism of the host, decreasing tissue glycogen, lactic acid and pyruvic acid, and specifically inhibiting succinic dehydrogenase; the relation of these changes to observed pathology is, however, not clear.

As has already been suggested, the bacterial toxins are not unique. Substances closely resembling the soluble toxins occur in the seeds of some of the higher plants and in the secretions of certain animals. Among the better

[10] Kun and Miller: Proc. Soc. Exp. Biol. Med., 1948, 67:221.

known toxins of plant origin (phytotoxins) are ricin (from the castor oil bean, *Ricinus communis*), abrin (from the jequirity bean, *Abrus precatorius*), and the similar substances, crotin and robin. The more familiar examples of similar poisons of animal origin (zootoxins) include snake venoms, the poisons of scorpions and spiders and an actively poisonous substance present in eel blood. The chemical behavior and physiological action of these poisons are strikingly similar to those of the bacterial exotoxins. Antitoxins have been prepared against a number of these substances and the antivenins have been used with considerable success.

The "Invasiveness" of Bacteria. It was early suggested that, in addition to toxins, some of the highly virulent bacteria which invade the host tissues with great rapidity are enabled to do so by the secretion of soluble substances termed *aggressins* by Bail[11] and *virulins* by Rosenow.[12] Although subsequent experiment has provided evidence which strongly supports this general concept, these terms have, at present, little more than historical significance. It has been found that virulence in terms of tissue invasion and destruction of the defense mechanisms of the host by bacteria may be subdivided into a number of component factors. The more important of these are the *hemolysins* and *leucocidins* which destroy the red and white blood cells, the *coagulases* and *fibrinolysins* which influence the formation and dissolution of blood clots and a *spreading factor* which produces a marked increase in the permeability of the host tissues. Although some of these properties are associated with virulence, others are not so correlated and their relative importance is uncertain. It is probable that the ability of a bacterium to invade the tissues is not dependent upon any single property but rather upon a combination of properties, including both those that are known and others that are as yet unknown.

Hemolysins. A variety of bacteria produce hemolysins, substances which bring about the dissolution of the red blood cells of higher animals. The bacterial hemolysins, which are to be distinguished from the immune hemolysins formed by an animal in response to the injection of red cells of another species (p. 290), are of two types, the so-called filterable hemolysins which are extra-cellular and may be separated from the bacterial cells by filtration, and the hemolysins which are demonstrated by the cultivation of bacteria on semi-solid media containing whole blood.

The *filterable hemolysins* are sometimes named after the bacteria which form them; a *streptolysin*, for example, is a hemolysin produced by streptococci, and a *staphylolysin* a hemolysin of staphylococci. Hemolytic activity is demonstrated by the addition of filtrate or whole culture to a suspension of washed erythrocytes in physiological salt solution; after a period of incubation the red cells are laked and hemoglobin appears free in solution. The relation of these hemolysins to other naturally occurring hemolysins such as saponins, the hemolysins present in snake venoms and the like, is uncertain.[13] The bacterial hemolysins appear to be proteins in nature, are inactivated by

[11] Bail: Arch. Hyg., 1905, 52:272.
[12] Rosenow: Jour. Inf. Dis., 1907, 4:285.
[13] For a general discussion of hemolysis see the exhaustive monograph of Ponder: *The Mammalian Red Cell and the Properties of Hemolytic Systems*. Protoplasma Monograph, No. 6, 1934.

heating (55° C. for thirty minutes), and are antigenic, *i.e.*, when injected into animals they stimulate the formation of antihemolysins.

That hemolytic activity is a property of a group of substances of bacterial origin rather than of a single substance formed by a number of bacterial species is indicated not only by differences in immunological specificity but also by the varied properties of the activity. Many hemolysins, for example, are oxygen-stable, but some, produced by the pneumococcus and certain strains of streptococci, are oxygen-sensitive, *i.e.*, they are active in the reduced form but inactive in the oxidized form, the oxidation-reduction being reversible at low temperatures.[14] Hemolysins further differ from one another in their heat and acid resistance and in the incubation time which precedes visible laking of the red cells. Differences in their activity on the erythrocytes of various species of higher animals may be marked; a given hemolysin, for instance, may
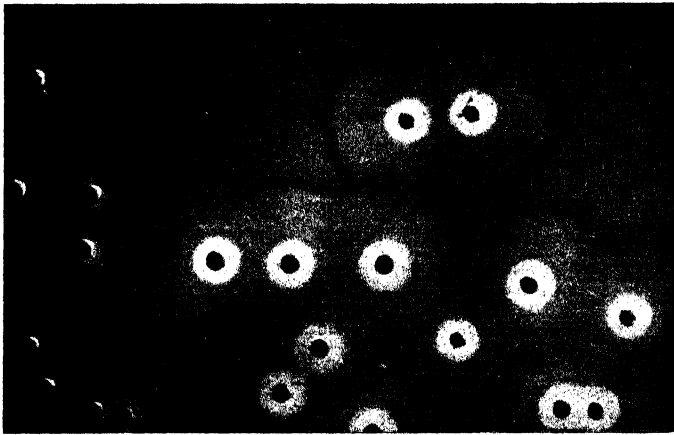
Fig. 28. "Hot-cold" hemolysis. *Staphylococcus aureus* on sheep blood agar. Produced by holding the plate alternately at incubator and refrigerator temperature. The small clear zones are produced in the incubator by the α lysin and the large zones in the refrigerator by the β lysin.

lyse sheep cells but not rabbit cells. In general the cells of the animal species from which a bacterium is isolated are more sensitive to its hemolysins than are the cells of other animal species.

A single bacterial strain may form more than one hemolysin. Certain strains of staphylococci form at least two hemolysins, one acting on both rabbit and sheep cells and bringing about a rapid lysis at 37° C., and the other acting only on sheep cells which are lysed after holding at room temperature overnight—a phenomenon which has been termed "hot-cold" lysis.[15]

The former is termed α lysin and the latter β lysin. The action of both can be shown in culture on sheep blood agar, the α lysin producing a zone of complete clearing on twenty-four hours' incubation and the β lysin producing a zone of darkening which becomes lighter and clear on subsequent refrigeration of the plate.[16] This phenomenon is illustrated in Fig. 28.

[14] *Cf.* Todd: Jour. Path. and Bact., 1934, 39:299..
[15] Glenny and Stevens: Jour. Path. and Bact., 1935, 40:201.
[16] Christie and Graydon: Australian Jour. Exp. Biol. & Med. Sci., 1941, 19:9.

Similarly, some strains of streptococci produce two hemolysins, one of which is relatively heat-stable but oxygen-sensitive and the other oxygen-stable but heat-sensitive.[14] It has been shown by Bernheimer[17] that streptolysin O, the oxygen-labile hemolysin, has a toxic action on the isolated frog heart. This cardiotoxic action is of particular interest in that it appears only on second application following a first or sensitizing application which releases an inhibitor and leaves the tissue susceptible to the action of the cardiotoxin.

The production of hemolysins is favored by inclusion of serum in the broth in which the microorganisms are cultured, and it is of some interest that the activity of the hemolysin formed in such media is most marked on the red cells of the animal species from which the serum was derived.[18] The hemolytic activity of a culture may be transitory, probably because of inactivation of formed hemolysin. Some hemolysins lose their activity when incubated at 37° C. for two hours or more; for example, de Kruif and Ireland[18] found a maximum hemolytic titer after eight hours' incubation of streptococcus cultures which declined rapidly thereafter so that in many cases no activity could be detected in filtrates of fourteen-hour cultures.

Blood-Plate Hemolysis. The colonies of some bacterial species on blood agar produce visible changes in the medium immediately surrounding the colony which are termed "hemolysis." Two general types of change are observed, one designated as α or green hemolysis, in which the bacterial colony is surrounded by a zone of greenish discoloration, and the other, β-hemolysis, in which the zone around the colony is clear and uncolored, in contrast with the red opacity of the medium. According to Brown[19] the zone of discoloration of α-hemolysis is surrounded by a very narrow clear zone, but this is not apparent except upon careful examination. Microscopic examination of the green zone shows the presence of many discolored corpuscles, but in β-hemolytic zones corpuscles cannot be found.

The relation between blood-plate hemolysis and the filterable hemolysins is usually regarded as uncertain, for, although microorganisms hemolytic on blood plates frequently do not appear to produce filterable hemolysin, some workers maintain that, since filterable hemolysins are difficult to demonstrate, failure to find them is not significant. It would appear that the processes involved are different; in the case of the filterable hemolysins the permeability of the red cell is altered and the hemoglobin escapes into the surrounding fluid, while in blood-plate hemolysis the pigment is broken down to green or colorless compounds. In the case of the cholera vibrio the two processes have been sharply differentiated,[20] since the identification of this organism is dependent in part on the hemolysis of goat erythrocytes in suspension (Grieg test); though the true cholera vibrio is negative to this test, some strains show hemolysis on blood agar plates. Van Loghem has distinguished between the hemolysis of goat erythrocyte suspensions and what he has termed hemodigestion on blood agar. On the other hand, there appears to be a close association between β-hemolysis and streptolysin O. Green hemolysis was formerly thought

[17] Bernheimer and Cantoni: Jour. Exp. Med., 1945, 81:295, 307.
[18] de Kruif and Ireland: Jour. Inf. Dis., 1920, 26:285.
[19] Brown: Rockefeller Institute for Medical Research, Monograph No. 9, 1919.
[20] Especially by van Loghem: see Centralbl. f. Bakt., I Abt. Orig., 1926, 100:19.

to be due to the formation of methemoglobin and associated with the formation of hydrogen peroxide, but it has been found[21] that the green substance is an iron-containing derivative of hemoglobin very possibly formed by reduction. The mechanism of β-hemolysis is not well known, but in the case of staphylococcal hemolysins, the hemoglobin is not destroyed; according to Christie and Graydon[16] the clearing around the colony is due to migration of the liberated hemoglobin. As in the case of the filterable hemolysins, hemolysis on blood agar is frequently species-specific; a higher proportion of bacterial strains will show hemolysis on blood from one animal species and not on that of another.

The relation of ability to form hemolysin and virulence is obscure. Although among some of the parasitic bacteria such as streptococci and staphylococci, virulence is associated with hemolytic activity, a number of saprophytic bacteria also produce hemolysins. On the other hand, it has been reported that in staphylococcus infections of the udder, the hemolytic strains are much more irritating than the non-hemolytic organisms. It might be expected that infection with actively hemolytic bacteria would be accompanied by anemia and hemoglobinuria but this is not the case, as a rule; infections with *Clostridium welchii*, however, are frequently characterized by a gross blood destruction and consequent severe anemia and jaundice which is due to the continuous release of the hemolytic α-toxin (p. 581). It is also not unlikely that hemolysins may exhibit toxicities other than that of the lysis of erythrocytes, as, for example, in the case of the cardiotoxic action of streptolysin O noted above.

Leucocidins. A number of bacteria, notably staphylococci, streptococci and pneumococci, form leucocidins, substances which kill and, in some cases, lyse, polymorphonuclear leucocytes. The death of the leucocytes results in the inability of these cells to reduce methylene blue, a phenomenon made use of by Neisser and Wechsberg[22] and others to demonstrate the activity of these substances. A direct microscopic method has been introduced[23] which has shed some light on the effect of these substances on the white blood cell. Human and rabbit leucocytes in contact with leucocidin become spherical; the nuclei fragment and the cells may, with some leucocidins, eventually burst or, with others, remain intact.

The amount of leucocidin produced by one bacterial species may vary widely from one strain to another, and a single strain may produce more than one leucocidin. The detection of more than one leucocidin is dependent upon differences in thermostability and in the kind of leucocytes attacked; one staphylococcal leucocidin, for example, affects both rabbit and human leucocytes, while another is active only on rabbit cells. In general, the leucocidins closely resemble the filterable hemolysins in that they are antigenic, variable in their heat resistance, etc., and, in some cases, may be identical with hemolysins. The α-hemolysin of staphylococcus, for example, is a leucocidin.[24] They may not be strain-specific; some of the staphylococcal leucocidins, for example,

[21] *Cf.* Anderson and Hart: Jour. Path. and Bact., 1934, 39:465.

[22] Neisser and Wechsberg: Ztschr. Hyg. u. Infektionskr., 1901, 36:299.

[23] Valentine: Lancet, 1936, i:526. It should be pointed out that it is claimed by some workers that the leucocidin tested by one method is not the same as that tested by the other.

[24] Wright: Lancet, 1936, i:1002.

are immunologically similar if not identical regardless of the bacterial strain from which they are derived.[25]

The part played by the leucocidins in the virulence of a bacterium is obscure. Theoretically, it should be of some advantage to an invading microorganism to be able to destroy these phagocytic cells and thereby break down, to some degree, the defenses of the body. The polymorphonuclear leucocytes (heterophils) are not, however, the most important cells in the phagocytic destruction of bacteria (p. 294), and the effect of these substances on the other phagocytic cells, the mononuclears, the histiocytes and others is not known. As in the case of the hemolysins, the leucocidins of bacterial origin must be differentiated from the immune leucocidins, those produced by the animal body in response to the injection of leucocytes from another species.

Coagulase[26] and Fibrinolysin. The formation of blood clots is accelerated by a substance, coagulase, formed by some bacteria, and the clots formed, either with or without the acceleration of this substance, may be dissolved by other substances of bacterial origin, the fibrinolysins. The production of coagulase, is, so far as present information is concerned, confined to the staphylococci for the most part, and may be causally related to the thrombi that are common in infections with these organisms. Occasional strains of other organisms such as *Pseudomonas pyocyaneus, Bacterium prodigiosum, Bacterium coli* and *Bacillus subtilis* have, however, been observed to accelerate the clotting of blood. Coagulase activity is demonstrated by the addition of bacteria to citrated or oxalated blood plasma (usually rabbit), which gels within three hours. Cellfree coagulase has, however, been prepared from plasma cultures of *Staphylococcus aureus.*[27] Coagulase is regarded by some as a precursor of a thrombinlike substance and requiring an activator present in some, though not all, plasmas.[28] Unlike coagulase, the thrombin-like substance is thermolabile. The clotting produced by coagulase is not, however, inhibited by "antithrombins" such as heparin or hirudin. The activity is not filterable and is relatively thermostable; it is only partially destroyed by exposure to 100° C. for thirty minutes. Other properties such as precipitation with alcohol and acid and ammonium sulfate and inactivation with trypsin and pepsin suggest a protein nature. Its antigenicity is uncertain. The relationship of coagulase to virulence is obscure, although it is often said that there is no association between the two. It would seem that the property of accelerating the clotting of blood might, on the one hand, be unfavorable to further invasion of the host tissues by a microorganism forming this substance, and, on the other, serve as a temporary protection against the defenses of the host. Hale and Smith[29] have observed that coagulase-producing staphylococci are less susceptible to phagocytosis in the presence of plasma than are coagulase-negative strains, presumably a result of coating the bacteria with a film of coagulum.

[25] For a general study of these substances see Todd: Brit. Jour. Exp. Path., 1942, 23: 136.

[26] For a summary of information regarding coagulase see the review by Blair: Bact. Rev., 1939, 3:97.

[27] Lominski: Nature, 1944, *154*:640.

[28] Smith and Hale: Brit. Jour. Exp. Path., 1944, 25:101.

[29] Hale and Smith: Brit. Jour. Exp. Path., 1945, 26:209; *ibid.*, 1947, 28:57.

· *Fibrinolysin.*[80] Perhaps more important to the virulence of a bacterium is the ability to dissolve formed blood clots through the agency of fibrinolysin. It has been observed that if a small amount of bacterial culture is mixed with oxalated plasma and the latter allowed to clot by the addition of calcium chloride, the fibrin clot may be dissolved within a short time as a result of bacterial activity. The activity may be titrated by determining the highest dilution that will lyse a fibrin clot in one hour at 37° C. At present it appears that the ability to bring about this lysis is confined to certain groups of streptococci, although other bacteria such as staphylococci, the plague bacillus and others have been described as fibrinolytic. Like the hemolysins, the fibrinolysins appear to exhibit some degree of specificity with respect to the source of the fibrin; streptococci isolated from human infections, for example, will lyse clotted human plasma more rapidly than plasma clots from other animals. Fibrinolysin appears to fall into the same general category as the toxins, hemolysins and the like in that it is found in culture fluid freed from bacterial cells, appears to be protein in nature, and is antigenic. Fibrinolysin is, however, distinct from these other substances; although a high correlation has been observed between the occurrence of filterable hemolysin and fibrinolysin, in many cases a bacterial strain is able to bring about the dissolution of erythrocytes but not solution of clots, and *vice versa.*

The nature of the lytic process has been of some interest, and it has been found[31] that fibrinolysin is an activator of an inactive proteolytic enzyme or "lysin factor" present in the serum. Thus the term fibrinolysin is a misnomer, and possibly the active enzyme should be termed "plasmin." In any case, the lytic process is accompanied by an increase in amino nitrogen and other manifestations of proteolysis.

Fibrinolysin is antigenic and fibrinolysins from different strains of bacteria appear to be very similar if not identical immunologically. Antisera specifically inhibit the activity and the occurrence of anti-fibrinolysin in the serum has been useful as an indicator of past infection with fibrinolysin-positive streptococci.

It should be noted here that a number of bacteria exhibit a tendency to inhibit the clotting of plasma, a property that may be demonstrated by delaying the addition of calcium to the oxalated plasma; when calcium is added after a period of preliminary incubation, the clot fails to form. This property has been most often demonstrated with cultures in dextrose broth, and in some cases, at least, the failure of the plasma to clot is a result of the presence of lactic and other acids formed in the fermentation.

Fibrinolysin appears to be closely associated with virulence and particularly with the ability to invade the body tissues. Since one of the first reactions of the body to tissue destruction is the formation of blood clots which tend to wall off and isolate the infected region, it is not surprising that a bacterium capable of lysing these clots should show marked tendencies to extensive tissue invasion. The streptococci are among the most invasive of the pathogenic forms, and this

[80] See the review by Tillett: Bact. Rev., 1938, 2:161.
[31] See Christensen: Jour. Gen. Physiol., 1945, 28:363, 559; Holmberg: Arkiv Kemi Mineral. Geol., 1944, *17A*:8.

phase of their virulence may be attributed in part to the formation of fibrinol-ysin.

Hyaluronidase (Spreading Factor, Invasin).[32] It has been found in recent years that the permeability of the tissues is remarkedly increased by a factor, often called the *Duran-Reynals factor* after its discoverer, present in certain mammalian tissues, notably the testes. Bacteria, vaccinia virus, and substances such as toxins, india ink and the like, diffuse rapidly from the site of inoculation when injected in conjunction with extracts containing this factor. Strains of staphylococci and streptococci that have no pronounced invasive powers may, in this way, be rendered highly invasive. This factor is also present in a number of bacteria notable for their invasive properties, such as certain strains of staphylococci and streptococci, pneumococci and certain of the obligate anaerobes such as the bacillus of gas gangrene. Among some of these organisms there is some degree of association between content of the Duran-Reynals factor and virulence; the non-invasive strains of staphylococci noted above contain little or none of this factor but become invasive when it is supplied, while the invasive strains produce the factor themselves. This sub-stance has been found to be an enzyme, hyaluronidase, whose substrate is hyaluronic acid, a mucopolysaccharide consisting of acetyl glucosamine and glucuronic acid, which acts as a cement substance of the tissues and is found in synovial fluid and elsewhere in the body. The action of the enzyme in de-creasing the viscosity of hyaluronic acid by hydrolysis is responsible for facili-tating the penetration of the tissues by bacteria that produce it. The effect on the tissues is temporary in that, following inoculation of preparations of the enzyme, the dermal barrier is restored partially in twenty-four hours and com-pletely in forty-eight hours.[33] It is of some interest that hyaluronic acid is also found in the capsular substance of some strains of streptococci. Such strains do not produce hyaluronidase and in its presence are denuded of cap-sules and more readily phagocytosed (see below). The ability of such strains to invade the tissues does not, of course, depend upon their elaboration of hyaluronidase. The activity appears to be antigenic, in that it is neutralized by antisera, but immunologically distinct from different sources; it has been sug-gested that the enzyme is combined with different proteins in different organ-isms.

Hyaluronidase is antagonized by an enzyme present in normal blood plasma which has been called anti-invasin I. An enzyme in bacteria, pro-invasin I, de-stroys anti-invasin I, and another enzyme in plasma, anti-invasin II, destroys pro-invasin I. It has been suggested that a balance between all these in the host-bacterial system determines whether or not invasion of the tissues will occur.[34]

Capsules. The remarkable association between the presence of a capsule on a pathogenic bacterium and its virulence has been discussed elsewhere (Chapter 6). The capsular material, generally polysaccharide in nature al-

[32] *Cf.* the reviews by Duran-Reynals: Bact. Rev., 1942, 6:197; and by Meyer: Physiol. Rev., 1947, 27:335.

[33] Hechter: Proc. Soc. Exp. Biol. Med. 1948, 67:343.

[34] Haas: Jour. Biol. Chem., 1946, *163*:63, 89, 101.

though nitrogen and amino acids may be present, is not in itself toxic and cannot be regarded as analogous to toxins, hemolysins and similar substances. Rather the capsule appears to function as a defensive mechanism of the bacterium against the phagocytic activity of the leucocytes. Encapsulated bacteria may be ingested by a white blood cell but, instead of being killed and digested, remain within the phagocyte for a time and then may be extruded in a viable condition. The ability of an encapsulated bacterium to resist phagocytic destruction may, in fact, result in a wider distribution of the microorganism than it might otherwise attain, through transport within the phagocytic cell. It is of some interest in this connection that non-encapsulated, avirulent pneumococci are highly virulent for rabbits deprived of their leucocytes.[35] It is perhaps suggestive that the polypeptide material making up the capsule of the anthrax bacillus contains d($-$) glutamic acid[36] (p. 556). Since proteolytic enzymes attack only polypeptides built up from amino acids of the *l*-series, possibly an encapsulated anthrax bacillus would be highly resistant to the digestive enzymes of a phagocytic cell.

Whether the association of virulence and capsule formation may be entirely accounted for on the basis of bacterial defense against phagocytic destruction is not entirely certain, but such defense undoubtedly plays an important part. The presence of antibodies to the capsular substance breaks down this bacterial resistance, and immunization to the capsular material of the pneumococcus, for example, produces just as high a degree of immunity to pneumococcus infection as immunization to the entire bacterium.

Miscellaneous Factors. In addition to these more or less well defined and better known factors, a number of bacteria have been reported to produce substances which may be associated with virulence. Such, for example, are the necrotizing factor, or necrotoxin, produced by some staphylococci, which kills tissue cells; a hypothermic factor produced by Shiga dysentery bacilli, which lowers body temperature; an edema-producing substance formed by pneumococci; substances associated with the endotoxins of some of the enteric bacteria which affect the blood sugar levels of animals, and so on. Unfortunately, the discontinuous character of present information does not support any satisfactory generalization regarding bacterial virulence, but it seems clear that a pathogenic bacterium may have at its disposal a series of mechanisms, in combination peculiar to itself, which make possible a successful invasion of the tissues of the host.

In this connection it should be noted that bacterial virulence may be apparently enchanced by certain substances not of bacterial origin. The presence of silica in the lungs, for example, predisposes materially to pulmonary tuberculosis; the injection of calcium salts with tetanus bacilli aids, in some unknown manner, in the establishing of a nidus of infection. The suspension of bacteria, such as meningococci or typhoid bacilli, in solutions of gastric mucin markedly enhances their virulence, or, more precisely, interferes with the defense mechanisms of the host. It has been found that in the case of the

[35] Rich and McKee: Bull. Johns Hopkins Hosp., 1939, 64:434.
[36] Bruckner and Ivanovics: Ztschr. f. physiol. Chem., 1937, 247:281; Ztschr. Immunitäts., 1937, 90:304; *ibid.*, 1937, 91:175; Naturwissenschaften, 1937, 25:250.

meningococcus the mucin interferes with the bactericidal action of the body fluids and, when mixed with peritoneal fluid, provides a medium for its growth.[37]

Relative Pathogenicity of Bacteria. From the foregoing considerations it will be clear that the virulence of a bacterium is an expression of the efficacy of the aggregate of offensive mechanisms that it may possess. It is to be expected, then, that the bacteria constitute a continuous series of types ranging from those which are unable to infect higher organisms, such as the autotrophic forms, to those which produce a fulminating, widely disseminated and often fatal infection. The harmless saprophyte, *Bacillus subtilis,* for example, has been found to cause, on occasion, an eye infection in human beings (see also p. 565), and the saprophytic microorganism, *Bacterium prodigiosum* which is frequently used as a test organism in part because of its non-pathogenicity, has been found to produce disease following inhalation.[38] Somewhat more pathogenic is *Clostridium botulinum,* an organism which is also a saprophyte but which produces a highly potent soluble toxin. The bacterium is itself unable to infect the animal body; the toxin is formed outside the body and gives rise to disease only when ingested in a preformed state. *Clostridium tetani* is one step higher in the scale of pathogenicity; this microorganism resembles the botulinus bacillus in that it is essentially a saprophytic form that produces a soluble toxin. In this case, however, the bacterium is able to establish a nidus of infection in the tissues, albeit in a strictly localized area, and produce the toxin whose absorption gives rise to the symptoms of the disease. The diphtheria bacillus likewise generally produces only a local infection from which its toxin diffuses into the animal body but, unlike the tetanus bacillus, does not lead a saprophytic existence in nature but must maintain a close association with its host. The highly invasive bacteria, such as certain stains of streptococci and staphylococci, are, however, disseminated rapidly throughout the body, presumably because of their armament of fibrinolysins, spreading factor and the like.

A variety of types of infection may, therefore, be distinguished. A bacterium having but small ability to spread through the tissues produces a *localized* or *focal* infection. Such, for example, are the abscesses at the roots of the teeth, the infection of the heart valves in bacterial endocarditis, etc. A local infection is not necessarily of small consequence; not only may the local tissue destruction be highly significant to the host, as in pulmonary tuberculosis, but, when the microorganism produces a soluble toxin, a poisoning or *toxemia* results, as in diphtheria or tetanus.

Many pathogenic bacteria exhibit a tendency to localize in one tissue in preference to another, sometimes termed *elective localization;* the meningococcus, for example, most frequently localizes in the central nervous system, as do the so-called "neurotropic" viruses, and some of the streptococci show similar preferences for the joints. In some cases the tendency to localize is so marked that a pathogenic bacterium is infectious by one route but not by another; microorganisms such as streptococci, pneumococci, the tetanus bacillus and

[37] MacLeod: Amer. Jour. Hyg., 1941, Sec. B. 34:51. See also Olitski: Bact. Rev., 1948, 12:149.
[38] Paine: Jour. Inf. Dis., 1946, 79:226.

others are relatively harmless when swallowed but regularly produce infection when injected into the tissues of a susceptible animal. Others, such as the organisms causing typhoid fever, cholera, the dysenteries and other enteric infections, are harmless when rubbed into the abraded skin but promptly produce disease when swallowed by man.

Bacteria present in a primary focal infection may spread by metastasis to set up multiple secondary foci, a condition known as *pyemia.* Other bacteria may multiply in the blood stream, a condition known as *bacteremia* or *septicemia;* the invading bacteria may become widely disseminated through the capillaries of the tissues with the production of a fulminating, *generalized infection.*

Infection is, however, in part a function of the defensive mechanisms of the host, and in some cases a bacterium capable of further spread is held in check by these defenses and sets up a *latent infection* which may flare up when resistance is reduced. Such an inconclusive outcome may result in the *carrier state,* a condition in which a virulent bacterium, fully capable of producing disease, is harbored by a healthy and unaffected host. In this latter instance, however, the resistance of the host is not infrequently specific and a consequence of previous immunization.

Mixed and Secondary Infections. It has long been known that an individual might be attacked by two or more infective agents at one time. Diphtheria and scarlet fever, syphilis and gonorrhea, pneumococcus pneumonia and typhoid fever, are combinations by no means unknown. It is possible that in some cases the different infections may originate nearly simultaneously, but such an occurrence is probably not common. Usually one infection precedes another, and the second is very frequently a more or less direct outcome of the first. Infection with certain microorganisms predisposes to secondary infection with the pneumococcus; acute tuberculosis may develop during an attack of measles; streptococcus invasion of the lung tissues is not uncommon in pulmonary tuberculosis. The secondary invader is commonly present in the host, but seems incapable of initiating an infection until the host defenses are weakened by the primary disease. Certain microorganisms that can cause primary infection are also frequently found as secondary invaders; pneumococci and streptococci are preeminent in this respect, and show a remarkable capacity for invading the body in the wake of other microorganisms.

Mixed infections of a somewhat different sort are those in which the principal pathogenic organism is accompanied by auxiliary bacteria, or, as some French bacteriologists have called them, *accomplices,* which by their presence influence the virulence of the chief infectious agent without themselves taking any very active part in the infectious process. Such cooperation is, of course, an instance of bacterial synergism which has been discussed previously. The aerobic bacilli which usually enter a wound along with tetanus bacilli probably facilitate the growth of the latter by providing anaerobic conditions. In other cases of mixed infections the invading bacteria may not influence one another directly but only indirectly in a joint breakdown of the defenses of the host; such is presumably the case in mixed infections of diphtheria bacilli and streptococci, and there is reason to believe that such a mixed infection is more severe than an infection with diphtheria bacilli alone—possibly spreading factor

formed by the streptococci increases the permeability of the tissues to the toxin formed by the diphtheria bacilli.

RESISTANCE

It is already apparent from the foregoing considerations of bacterial virulence that, although the ability of a microorganism to produce disease is conditioned by a series of mechanisms originating with the bacterium, pathogenicity must be evaluated in terms of the resistance of the host. As a rule, a pathogenic bacterium is limited to a small number of hosts; bacteria pathogenic for animals are not ordinarily pathogenic for plants; very few of the bacteria that can infect mammals are also pathogenic for cold-blooded animals; some are even restricted to the tissues of a single species. Resistance, like virulence, is made up of many factors, some of which are known either in more or less specific form or in the terms of generalities that serve as a cloak for ignorance; others are, in all probability, as yet unsuspected. Resistance to infection is, in a sense, somewhat more complex than virulence for, as will appear, not only are there specific barriers to infection variable with respect to species and even from one tissue to another in the body of a single animal, but the efficiency of these barriers is also a manifestation of general physiological well-being and hence they are subject to extrinsic or environmental influences.

The differentiation of resistance from natural immunity (p. 327) cannot be made with precision, for the two merge with one another and with acquired immunity without sharp lines of demarcation. In a general way they may be separated on the basis of specificity, resistance being non-specific with respect to the invading microorganism while immunity is sharply specific for a given parasite. The non-specific cellular response (p. 322) is intermediate between the two since the specific cellular immunity of the immunized animal is qualitatively the same, differing only in that it is markedly accentuated.

As in the case of virulence, the better known components of resistance are best considered one by one.

Species, Racial and Inherited Resistance. Species of higher organisms differ greatly from one another in their resistance to any given disease, a fact that has been suggested earlier in connection with the experimental reproduction of disease. In many cases resistance to infection is relative, for disease may sometimes be produced by the administration of massive doses of bacteria to a resistant animal, but in others it appears to be absolute. Man is, for example, apparently completely immune to cattle plague, and many of the lower animals are equally resistant to some diseases of man. In general the factors underlying differences in species resistance are unknown, but in a few cases body temperature or differences in anatomical structure have been found to account for the observed variation. Pasteur's classic experiment in which he rendered the naturally resistant hen susceptible to anthrax by chilling it in cold water, and the converse of this experiment, the production of anthrax in the resistant frog by raising its body temperature to 25° to 35° C., may be explained in terms of unfavorable body temperatures. Similarly, cold-blooded animals are not susceptible to human tuberculosis and warm-blooded animals are not infected by the tubercle bacilli of cold-blooded animals. The insuscep-

tibility of experimental animals such as guinea pigs and rabbits to the entero-toxin produced by some bacteria is possibly attributable to their lack of a vomiting mechanism. As indicated earlier, resistance to a given infectious agent is not necessarily associated with phylogenetic relationships, and there is no pattern from which the resistance or susceptibility of an animal can be predicted by logical processes; the tabulation of animals susceptible to a given disease represents information acquired largely by trial and error.

Domestic and Experimental Animals. Not only do species of higher organisms differ in their resistance to infectious disease but the races comprising a susceptible species likewise appear to differ among themselves. There are many instances of differences in resistance to infectious disease in varieties or strains of animals. The relative resistance of Algerian sheep to anthrax is well known and inbred Berkshire swine have been found to be highly resistant to brucellosis.[39] That this is a true racial immunity which is, as might be expected, inheritable, has been demonstrated by extensive experimental investigations with laboratory animals. The earlier studies of Wright and Lewis[40] showed that marked differences in susceptibility to tuberculosis existed between inbred families of guinea pigs, differences which were transmitted to the offspring. Later work, summarized by Webster,[41] Hill[42] and Greenwood *et al.*,[43] has been confined, for the most part, to studies on the susceptibility of mice to infection with *Salmonella typhi-murium* and similar bacteria; it has been shown that resistance to such infections may be raised or lowered by selective breeding, sometimes to a remarkable degree. Similarly, strains of mice differ in their susceptibility to murine typhus, and Lurie[44] has bred strains of rabbits resistant and susceptible to infection with tubercle bacilli. It is of interest that resistance to bacterial endotoxin may also be raised or lowered by selective breeding. Resistance does not behave as a simple Mendelian character but is to some extent specific in that a race having increased resistance to infection with one microorganism is not necessarily unusually resistant to another. For example, in Webster's work mice selected for resistance to infection with *Salmonella enteritidis* showed increased resistance to pneumococcus and Friedländer's bacillus infection, but were more susceptible to the virus of louping ill than the strain selected for susceptibility.

Considerable interest has attached to the mechanisms underlying inherited resistance and susceptibility, and in recent years suggestive results have been reported by a number of workers. Thus, in Lurie's[44] studies resistance was associated with low skin permeability as assayed by intradermal inoculation of india ink, increased rate and intensity of antibody (agglutinin) response, and the development of a high degree of hypersensitivity (see also p. 343). Gowen and Calhoun[45] have shown that in mice there is a marked correlation

[39] Cameron, Gregory and Hughes: Amer. Jour. Vet. Res., 1943, 4:387.
[40] Wright and Lewis: Amer. Naturalist, 1921, 55:20.
[41] Webster: Medicine, 1932, *11*:321.
[42] Hill: Medical Research Council, Special Report Series, No. 196, 1934.
[43] Greenwood, Hill, Topley and Wilson: Medical Research Council, Special Report Series No. 209, 1936.
[44] Lurie: Amer. Rev. Tuberc., 1941, 44: Suppl.
[45] Gowen and Calhoun: Jour. Inf. Dis., 1943, 73:40.

between numbers of leucocytes and resistance to mouse typhoid. Similarly, Severins, Roberts and Card[46] have found that resistance and susceptibility of breeds of chickens to *Salmonella pullorum* infection is associated with numbers of lymphocytes, and Oakberg[47] has observed differences in liver and spleen in resistant strains of mice associated with the ability of macrophages to digest phagocytosed bacteria. Differences in susceptibility may, of course, be a reflection of corresponding differences in immunizability, *i.e.*, ability to respond to antigenic stimuli with antibody production. Scheibel,[48] for instance, has been able to divide strains of guinea pigs into good and poor producers of diphtheria antitoxin on immunization.

Races of Man. The relative resistance of races of man to infection has been the subject of considerable interest and such investigation as has been possible. Under ordinary circumstances in this country the non-white races are much more susceptible to infectious disease than the white race. There are, however, certain exceptions. Thus, the influenza epidemic of 1918 appears to have had a greater impact upon the death rate for white youths than upon that for the non-white population in the same age group. Similar exception may be noted in the case of Baltimore Negroes who showed a lower ratio of clinical diphtheria to immunizing infections than corresponding white children.[49] It is quite generally recognized, too, that the Negro has a remarkable degree of resistance to erysipelas, and the more favorable response of the Negro to all forms of treatment for gonorrhea is well known.

Special interest has attached to the white and non-white tuberculosis death rates, both crude and age-specific (p. 643). Whether the observed high mortality in the non-white represents a racial susceptibility or is entirely a reflection of economic status has been the subject of considerable discussion. Studies on this question made in the Army (p. 644) would seem to indicate a true racial difference.

There also appear to be differences between the less well-defined "races" of man. There is evidence that the Irish are less resistant to tuberculosis than certain other elements of the American population, such as the Italians.[50] On the other hand, the Jewish race is considered by many to be relatively resistant to tuberculosis; in spite of a high incidence of infection, the mortality is very low.[51] To what degree the evidence supports the hypothesis that races[52] of man differ in their susceptibility to this and other diseases, such as pneumonia, is problematical, for adequate control of the environmental factors is difficult if not impossible. However this may be, twin studies on tuberculosis indicate the operation of genetic factors in the resistance of man to this disease,[53] and

[46] Severins, Roberts and Card: Jour. Inf. Dis., 1944, 75:33.
[47] Oakberg: Jour. Inf. Dis., 1946, 78:79.
[48] Scheibel: Acta Path. et Microbiol. Scand., 1943, 20:464.
[49] Frost: Jour. Prev. Med., 1928, 2:325.
[50] Guilfoy: Quart. Publ. Amer. Stat. Assn., 1907, 10:515; Dublin: Amer. Econ. Rev., 1916, vol. 6, no. 3; Dublin and Baker: Quart. Publ. Amer. Stat. Assn., 1920.
[51] For example, see Drolet: Amer. Rev. Tuberc., 1924, 10:280.
[52] In a number of these studies racial stocks were determined for first generation immigrants on the basis of parental birth place.
[53] *Cf.* Kallman and Reisner: Amer. Rev. Tuberc., 1943, 47:549.

the question is rather one of the occurrence of practically significant genetic segregation.

A given disease, however, may be relatively mild in its effect on races of man which have been in contact with it over a long period of time, but assume a highly virulent form in other races to which it is new. Measles, for example, a mild disease to civilized man, has been a scourge to certain primitive races. In other cases, diseases originally highly virulent have become apparently less so with the passage of time; leprosy is not as widespread as it was in biblical times, and syphilis is a considerably milder disease today than it was in the sixteenth century. Phenomena such as these have been taken by some to indicate the development of a racial immunity through a selection of more resistant individuals, and by others to suggest an adaptation of the microorganism accompanied by a loss of virulence. At the present time, it is not possible to differentiate sharply between these two; possibly both effects are operative.

It should be pointed out in this connection that what might be called a pseudo-racial immunity may be manifested by a race in close association with a given infective agent. Many individuals have the disease, the survivors are immune, and this immunity is passively transferred to the offspring (see p. 333), who are infected before this passive immunity entirely disappears and consequently have the disease in a mild form but become solidly immune. The immunity is passively transferred to the third generation, and the process continues *ad infinitum* as long as the race is in contact with the disease. Some such mechanism as this appears to account for the apparent racial immunity of the West African Negro to yellow fever; the immunity of adults arises as a result of mild infection in childhood.

Age. The effect of the age of an animal on its resistance to infections disease is variable, being clearly apparent in some instances and indistinguishable in others. In many cases there is a direct relation between age and resistance, the young being more susceptible than the older individuals. The suitability of the developing chick embryo as a menstruum for the cultivation of certain filterable viruses to which the chicken is apparently completely insusceptible, and the results of the experiments of Woolpert and his colleagues[54] on the infection of the guinea pig fetus, may be regarded as examples of an exaggerated susceptibility of the immature organism. The marked susceptibility of the guinea pig fetus to tuberculosis drops off after birth, and young, mature and old animals have been found to be progressively more resistant to this infection. Similar results have been observed with other infections such as equine encephalomyelitis in mice, St. Louis encephalitis in mice, and in the resistance of the rat to diphtheria bacilli and diphtheria toxin.

It is well known that the very young child does not respond well to active immunization; Sauer,[55] for example, has reported poor results in active immunization against whooping cough, and antidiphtheria immunization of the newborn is not successful. This inadequate immune response is undoubt-

---

[54] *Cf.* Woolpert: Amer. Jour. Path., 1936, 12:141; Woolpert *et al.*: Jour. Exp. Med., 1938, 68:313; Gallagher and Woolpert: Jour. Exp. Med., 1940, 72:99; Dettwiler, Hudson and Woolpert: Jour. Exp. Med., 1940, 72:623.

[55] Sauer: Amer. Jour. Path., 1941, 17:719.

edly at least partially responsible for infection of the very young with bacteria
ordinarily regarded as harmless saprophytes. Cass,[56] for example, has reported
epidemic infection of the newborn (in a maternity ward) with *Bacterium aero-
genes* which took a septicemic form, and the *Bacterium coli* infection of foals
known as scours is well known.[57] Complement deficiency in the newborn and
less active phagocytosis by Kupffer cells are possibly associated with this failure
to respond to antigenic stimuli and bacterial invasion.

The rise in resistance coincident with the development of an animal to ma-
turity has been interpreted by some workers as indicative of a "maturation
immunity" in which the ability to withstand infection is associated with the
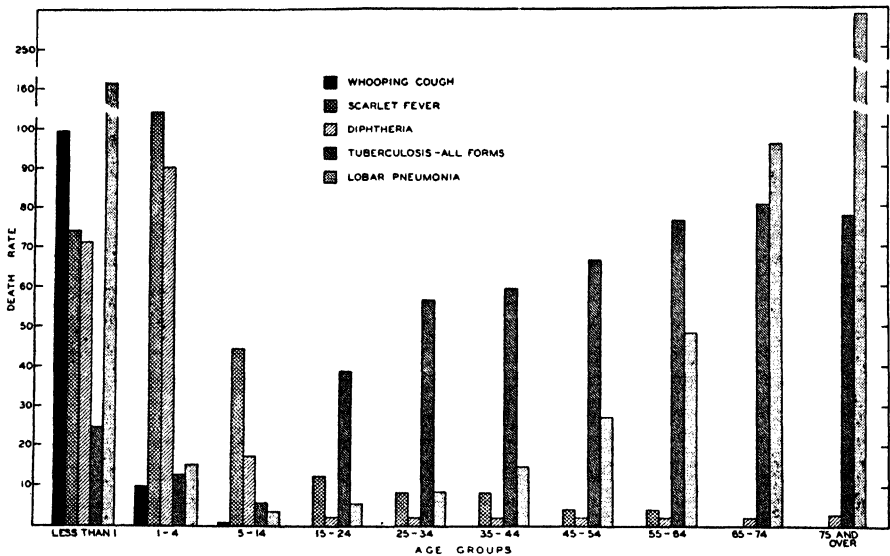
Fig. 29. The age incidence of certain infectious diseases. Note the predominance of the
diseases of childhood in the early years, the increase in tuberculosis in the young adult
years and the marked increase in lobar pneumonia with advancing age. Scarlet fever rate
multiplied by a factor of 40, and diphtheria rate by a factor of 10 for comparative purposes.
Data for 1940 from the reports of the Bureau of the Census.

ductless gland secretions. Experimental studies in which precocity has been
induced by the injection of androgens have, however, been disappointing, and
maturation immunity must be regarded as a hypothesis which is suggested by
certain clinical results but for which there is no sound experimental proof.

The well-known increased susceptibility of the aged to certain of the infec-
tious diseases is reflected not only in the incidence but also in the mortality
of infections such as pneumonia. The quantitative (exponential) relation
between the probability of death from a given disease and age was observed
many years ago (1825) and is known as Gompertz's law. The physiological
alterations underlying senile debility and mortality are probably in the
nature of an accumulation of degenerative changes.[58]

[56] Cass: Lancet, 1941, *i*:346.
[57] For instance, see a discussion by Wolfe and Dilks: Jour. Immunol., 1948, 58:245.
[58] Simms: Science, 1940, *91*:7.

In practice, the relation of age to resistance to infection is complicated by many factors. The human baby, for example, is relatively resistant to a number of infections for the first six months of life as a result of passive transfer of antibodies from the mother either via the placental circulation *in utero* or by means of colostrum after birth. Similarly, the increased resistance to a disease such as diphtheria which appears with physical development arises primarily as a result of inapparent infection rather than maturity alone. (The so-called natural antibodies are discussed on p. 329.)

Sex. A number of diseases exhibit a difference in sex incidence; pneumonia and epidemic meningitis, for example, are more common in males than females, while scarlet fever, typhoid fever and others occur somewhat more frequently in females. Except for a few years in the forty-one-year period 1900–1940, the female death rate for white youths has been consistenly lower than that for males, though the reverse has been true for the non-white population in this country. In the decade 1931–1940 the gap between the female and male in the non-white population was closed and in 1940 the female and male death rates for non-white youths were exactly the same at 5.0 per 1000. Corresponding rates for the white population were 1.4 for females and 2.0 for males. In the case of tuberculosis, variability in the age-specific mortality rates indicates a differential response on the part of the sexes (p. 644). A sex differential is also indicated by the aftermath of the 1918 influenza epidemic, which appears to have had a more unfavorable effect upon female mortality than that of males in the 15 to 24 age group, as indicated by the relatively high female death rate for all racial groups for several years after the epidemic.

It has been suggested that this difference between the sexes might result from resistance having some of the attributes of a sex-linked character, or, since differences in sex incidence are most pronounced during and after puberty, something in the nature of maturation immunity may be involved. There is no sound evidence to support the first of these hypotheses. Regarding the second a certain amount of experimental evidence has been reported, the most precise relating to skin permeability, which has been found to be greater in female rabbits than in males.[59] It has also been shown by Bennison and Coatney[60] that female chicks are significantly more susceptible to infection with the chicken malaria parasite, *Plasmodium gallinaceum*, than are males; treatment with male and female sex hormones did not affect the differences between the two. Any explanation of the observed sex differences must, of course, take into consideration other factors such as occupation, risk of exposure and the like.

Climate and Season. That both climate and the season of the year exert marked effects on the incidence and mortality of a number of infectious diseases is well known. In tropical climates, for example, the acute upper respiratory infections are not so common as in the temperate climates, but the dysenteries are more common in the tropics than in the temperate zones. The seasonal incidence of the infectious diseases is also common knowledge;

[59] Lurie and Zappasodi: Proc. Soc. Exp. Biol. Med., 1939, 42:741; *ibid.*, Arch. Path., 1942, 34:151.

[60] Bennison and Coatney: Science, 1948, *107*:147.

meningococcus meningitis occurs predominantly in the winter and spring, poliomyelitis in the late summer and early fall, and so on. One of the most striking relationships of season to incidence of disease is that of Asiatic cholera; the epidemic season coincides with hot weather and shows a remarkable correlation with precipitation and relative humidity.

In a great many instances the influence of climate and season upon the incidence of disease may be attributed to opportunities for transmission of the causative organism, such as crowding in poorly ventilated rooms, seasonal or geographic occurrence of an insect vector and so on, but in others the factors responsible for seasonal incidence are unknown.

In recent years specific evidence has begun to accumulate which substantiates the opinion that resistance varies with season, temperature and similar factors. For example, the intensity of the brain reaction of mice inoculated with St. Louis encephalitis virus and that of guinea pigs inoculated with endemic typhus virus have been found to have been the greatest in summer and the least in the winter over a period of years; mice adapted to existence in a moist heat have been found only one quarter as resistant to infection with hemolytic streptococci as those adapted to a cool environment; the morbidity and mortality rates in murine typhus infection in mice are affected by environmental temperature; and the resistance of mice to pneumococcus infection is similarly affected. It is not improbable that future investigation will yield systematic knowledge of the effect of climatic factors on susceptibility to disease.[61]

General Physiological Well-Being. Whatever resistance to disease an organism may possess by virtue of species, race and the like is profoundly influenced by its general physiological state. In general, resistance is at its height when the organism is functioning normally in every respect, and is reduced by a variety of factors which interfere with and alter the normal physiological state. In some cases previous infection may reduce resistance to such a point that infection with the less virulent bacteria may take place, as in the secondary infections; in others, functional disorders such as diabetes mellitus bring about a reduction in resistance to infection. More common, however, are the deleterious effects of inadequate diet and fatigue.

Nutrition. The relation between susceptibility to infection and faulty nutrition has been of considerable interest since the discovery of the vitamins and consequent study of the deficiency diseases, in some of which, such as xerophthalmia, infection plays a part. Attention has been directed particularly toward vitamins A (carotene) and C (ascorbic acid), and there is no doubt that a deficiency of the first of these substances results in a marked lowering of resistance. The reduced resistance appears to be associated with a reduced humoral immune response, but this is in part at least compensated for by an accentuated phagocytic response to infection. Diets qualitatively and quantitatively inadequate, not only with respect to other vitamins but in other ways also, may likewise predispose to bacterial infection.[62] Recently Cannon[63] has

---

[61] *Cf.* Mills: *Medical Climatology.* Charles C Thomas. Springfield, Ill. 1939.

[62] See the reviews of Robertson: Medicine, 1934, *13*:123; Clausen: Physiol. Rev., 1934, *14*:309; Jour. Amer. Med. Assn., 1935, *104*:793; Cowell *et al.*: Proc. Roy. Soc. Med., 1937, *30*:1039.

[63] Cannon: Jour. Bact., 1946, *51*:582.

emphasized the importance of adequate protein intake, and he and his co-workers have shown that depletion of the protein reserves of experimental animals by maintenance on low protein diets interferes markedly with antibody formation, *i.e.*, the synthesis of immune globulin. While antibody formation is significantly interfered with by moderate protein depletion, a severe depletion also interferes with the normal functioning of the cellular defense mechanisms as evidenced by reduced phagocytosis.[64]

Other than this, just how malnutrition brings about a reduction in resistance is not known; present information indicates that the effect is a general one. It is of some interest that this unfavorable effect is not limited to the undernourished individual but may be transmitted to offspring. Experimental studies have shown,[65] for example, that the diet of mice, genetically homogeneous in resistance to an infection, affects the resistance of offspring to a greater degree than the diet of the offspring themselves. In this connection, statistical studies[66] have suggested that the mortality rates of adult human life are in part a function of nutritive and other elements of the environment of childhood; in other words, the effect may be a delayed one, but the evidence on this point is not altogether indubitable.[67]

The marked reductions in resistance associated with inadequate diets are in no sense specific; resistance to infection in general is reduced, and there is no relation between lack of a single dietary factor and susceptibility to a particular infection. A number of attempts have been made to demonstrate such a relation; ascorbic acid, for instance, has some small capacity to neutralize diphtheria toxin, but vitamin C deficiency does not predispose to infection with the diphtheria bacillus any more than with other organisms, such as the tubercle bacillus.

Since inadequate diet, and vitamin A deficiency in particular, reduces resistance to infection, it has been supposed that resistance might be raised in hypervitaminosis. Experimental evidence, however, does not support this supposition; the normal level of resistance maintained by the organism on an adequate diet cannot be appreciably raised by the administration of vitamin A in quantities in excess of that normally required.

The apparently contradictory observation that resistance to infection with a number of the viruses is increased by inadequate diet has been made by a number of workers.[68] That it is not completely inconsistent with the lowered resistance associated with protein depletion and other dietary deficiencies is indicated by the consideration that the viruses are obligate intracellular parasites; possibly a healthy cell is of primary importance to multiplication of the virus and the depleted cell is an unfavorable medium for development, thus masking so to speak the lessened immune response associated with nutritional deficiencies.

Fatigue. It has long been known to the clinician that bodily rest is a valuable adjunct to the treatment of disease, and there is clinical evidence

[64] *Cf.* Mills and Cottingham: Jour. Immunol., 1943, 47:503; Guggenheim and Buechler: *ibid.*, 1948, 58:133.

[65] *Cf.* Church: Amer. Jour. Pub. Health, 1939, 29:215.

[66] *Cf.* Greenwood: Jour. Roy. Stat. Soc., 1936, 99:674.

[67] Cheeseman: Human Biol., 1938, 10:537.

[68] *Cf.* the summary of the literature by Kearney, *et al.*: Jour. Bact., 1948, 55:89.

which suggests that resistance to the initial infection may be reduced by excessive fatigue. Experimental evidence on this point is scanty and to some degree conflicting, but it is probable that the unfavorable effect of fatigue on normal physiological well-being is reflected to some extent in an increase in susceptibility to infection. The normal white rat, for example, is highly resistant to anthrax, but when exhausted by work in a treadmill becomes susceptible;[69] latent *Salmonella enteritidis* infections in the same experimental animal may be activated by fatigue to such a degree that the outcome is fatal.[70] Similarly, studies on human beings have indicated that an individual may be rendered transiently susceptible to the common cold by fatigue.[71]

Other mechanisms operative in the resistance associated with general physiological well-being are obscure. Studies on resistance to the common cold in population groups made by subjecting the incidence data to an analysis of variance among groups have strongly suggested that an as yet undefined constitutional factor is operative in the etiology of the clinical infection.[72] The studies of Locke[73] have indicated that the capacity to maintain effective circulation and the ability to withstand the effects of sudden temperature changes are associated with resistance to experimental infection. The adverse effects of sudden changes in temperature and humidity on the organism, reflected in changes in the nasal mucosa, are, perhaps, a manifestation of temperature shock. Attempts to associate such shock, vitamin deficiencies, fatigue and other elements of the non-specific resistance of health with specific defense mechanisms such as the capacity to form antibodies, have not been uniformly successful.

The External Defenses of the Organism. The cellular organization of the animal body is a closed system with respect to the outside environment from which it is separated by the skin, mucous membranes and intestinal mucosa. These structures, generally impermeable to particulate material of the size of bacteria, constitute the first line of defense against invading microorganisms and one that is, for the most part, highly effective. While mechanical obstruction contributes in no small part to the efficacy of these barriers, both skin and mucous membranes also play an active part in the protection of the organism against bacterial invasion, as will appear.

The Skin. As a rule, the unbroken skin presents a more or less impassable barrier to microorganisms. Bacteria are found normally on the skin between the superficial horny cells, but ordinarily are not able to penetrate deep into the tissues unless favored by some cutaneous injury, such as a wound or burn. The ducts of the sweat glands and the hair follicles are, however, vulnerable points, and experiment has shown that it is possible for bacteria to penetrate the skin through these channels.

The skin, however, is not an inert surface upon which bacteria may survive, but is, as experimental work of the last few years has shown, actively bactericidal. Arnold and his co-workers[74] found that bacteria which do not

[69] Charrin and Roger: Arch. Physiol. Norm. Path., 1890, 22:273.
[70] Boycott and Price-Jones: Jour. Path. and Bact., 1926, 29:87.
[71] Locke: Jour. Immunol., 1939, 36:365.
[72] Sargent, Lombard and Sargent: Amer. Jour. Hyg., 1947, 45:29.
[73] Locke: Jour. Immunol., 1939, 36:159, 172, 183, 365.
[74] *Cf.* Arnold, Gustafson, Montgomery, Hull and Singer: Amer. Jour. Hyg., 1930, *11*:345.

occur normally on the skin, such as *Bacterium prodigiosum,* the typhoid, colon and enteritidis bacilli, and hemolytic streptococci, are rapidly (in some cases within ten minutes) destroyed when swabbed on the clean skin of the palm of the hand. Possibly related is the fungistatic action of the free saturated aliphatic acids in the hair fat of adults.[75] The autosterilizing capacity of the skin is markedly reduced by dirt and is apparently a property of live skin; the skin of a cadaver dead only fifteen minutes showed little or no bactericidal activity. Bacteria normally present upon the skin, such as the white staphylococci, are not appreciably reduced in numbers when swabbed on the clean skin, a fact which probably accounts for their constant presence on the body.

The Conjunctivae. Bacteria and dust particles settling in the eyes are removed relatively rapidly by the mechanical flushing effect of the tears. The lacrimal secretions contain a substance, *lysozyme,* which is also present in certain tissue extracts and in egg white, which destroys some species of saprophytic bacteria. Fleming[76] who discovered lysozyme, described one species of bacteria, *Micrococcus lysodeikticus,* which is particularly susceptible to the action of this lytic substance; as high a dilution as 1:40,000 of tears has brought about complete lysis of these microorganisms. Lysozyme has been found to be identical with the avidin of egg white and has been isolated in crystalline form.[77] None of the pathogenic forms, however, appears to be unusually susceptible to the action of lysozyme, and it probably plays no important part in the protection of the organism against invasion.

The Nose, Nasopharynx and Respiratory Tract. Bacteria and other particulate material present in inspired air are rapidly removed by passage through the tortuous nasal passages lined with mucous membrane to whose moist surface they cling. In this way air is largely freed from bacteria in the upper respiratory passages, those that pass the larynx are caught in the bronchi and few reach the ultimate ramifications of the bronchioles. The process is so efficient that expired air contains almost no bacteria except those that are expelled in droplets by sneezing, coughing and talking.

The moist film which covers the mucosa of the upper respiratory tract and in which bacteria removed from inspired air and those arriving via the lacrimal secretions are embedded consists of mucus, a thin, highly viscid substance that, in a sense, constitutes a continuous web or membrane overlying the surfaces within the nose, sinuses, pharynx and esophagus. This film of mucus is in constant motion as a result of the activity of cilia which sweep the mucus and its bacterial content toward the oropharynx, where it is swallowed. The exchange of mucus is rapid; that covering the posterior two thirds of the nose is replaced every ten or fifteen minutes, while that over the anterior third is removed every hour or two.[78] Although mucus itself has no bactericidal activity, when combined with ciliary activity it constitutes a remarkably efficient means of ridding the upper respiratory passages of bacteria. Davies,[79] for instance, has shown that all particles above 7 μ in diameter

[75] Rothman, Smiljanic and Shapiro: Proc. Soc. Exp. Biol. Med., 1945, 60:394.

[76] Fleming: Proc. Roy. Soc., 1922, Series B, 93:306; Lancet, 1929, i:217; Proc. Roy. Soc. Med., 1932, 24: (Sec. Path.) 1. See review by Thompson: Arch. Path., 1940, 30:1096.

[77] Alderton, Ward and Fevold: Jour. Biol. Chem., 1945, 157:43.

[78] Cf. Hilding: Ann. Int. Med., 1932, 6:227.

[79] Davies: Proc. Roy. Soc., Ser. B, 1946, 133:282.

which are inhaled are retained in the upper respiratory passages of the rabbit, about half the particles 3 μ in diameter are similarly removed, and the remainder plus practically all those 1.5 μ in diameter and smaller penetrate into the lungs. Substantially the same results have been reported by Boyland, Gaddum and McDonald[80] with other experimental animals and man. The bacteria that penetrate the upper respiratory passages and lodge in the bronchi and bronchioles are probably phagocytosed by the fixed aveolar epithelial cells and the wandering leucocytes that enter the bronchioles and sacs, a process discussed at greater length elsewhere.

Lysozyme is, of course, present in the nasal mucus and it has been observed[81] that the normal serous secretion of the nose contains a virus inactivating agent (VIA) distinct from lysozyme. The activity is virucidal for the influenza and certain other viruses inactivated by sodium desoxycholate but what part it plays in resistance to infection is not clear.

The Mouth, Stomach and Intestinal Tract. The healthy mouth ordinarily contains great numbers of bacteria, but, except in the case of those organisms which have established themselves, this flora is a transient one, the microorganisms being constantly removed through a flushing action of saliva and as constantly supplemented by fresh contamination. Saliva is but mildly bactericidal,[82] and the removal of bacteria is practically entirely a mechanical process. The microorganisms flushed to the back of the mouth meet with those from the nose and, with them, are swallowed.

Bacteria reaching the stomach are subject to the strongly acid environment of the normal gastric juice, and there is no doubt that the great majority of them are destroyed there. Some do, however, reach the intestinal tract, perhaps because they are embedded in solid particles of food and thus protected or because they are able to withstand a short exposure to the bactericidal action of the gastric secretions. Generally, very few viable bacteria are found in the stomach, but the numbers of microorganisms increase in the small intestine with the rise in pH from the duodenum to the ileum. The large intestine contains great numbers of bacteria derived not only from the upper levels of the intestinal tract but also from the multiplication of bacteria present in the intestines as normal inhabitants. As in the respiratory passages, mucus plays an important part in the mechanical removal of bacteria. Here, however, the mucus does not form a uniform coating over the intestinal mucosa but is present as a meshwork. The villi free themselves from particles by movements which bring them in contact with the mucus to which the particles, including bacteria, adhere. The mucus, with the embedded microorganisms, is rolled up into small masses and moved outward by the peristaltic movements of the bowel.[83] Bacteria, then, which enter the mouth and upper respiratory tract are eventually extruded with the feces.

The Genital Tract. The normal genital tract is remarkably free from bacteria. The urethra in both male and female is normally sterile, a consequence,

---

[80] Boyland, Gaddum and McDonald: Jour. Hyg., 1947, 45:290.
[81] Burnet, Lush and Jackson: Brit. Jour. Exp. Path., 1939, 20:377; Francis: Science, 1940, 91:198.
[82] *Cf.* Van Kesteren, Bibby and Berry: Jour. Bact., 1942, 43:573.
[83] Florey: Jour. Path. and Bact., 1933, 37:283.

perhaps, of the flushing action of the slightly acid urine. The few bacteria that may be present are confined to the region of the meatus. The normal vaginal secretion is acid and is markedly bactericidal toward most species of bacteria.

Normal Flora. A microorganism somewhat better able to resist the defensive mechanisms of the host but at the same time unable, except when resistance is reduced to a low level, to invade the body tissues, may exist in conjunction with the host as a part of the latter's "normal flora." The staphylococci which are able to resist the bactericidal action of the skin are almost invariably present on these surfaces and are regarded as "normal" inhabitants. The scanty bacterial flora of the vagina, on the other hand, is composed almost entirely of aciduric bacteria; and the bacterial types present in the intestines are determined to a considerable extent by the type of food material present, *i.e.*, the diet of the host, and by the pH of the various intestinal levels. The composition of the intestinal flora is also affected to an indeterminate extent by antagonistic relationships among the bacteria present and potentially present. It has been shown,[84] for example, that some strains of coliform bacteria elaborate an antibiotic, colicin, which is effective against dysentery bacilli. Certain kinds of bacteria, such as lactobacilli, spirochetes, various cocci and the like, exist in the mouth in the interstices between the teeth and in and under tooth plaques and constitute a normal flora characteristic of this region. The bacteria commonly present in the nose and throat consist of still different forms, such as pneumococci, Friedländer's bacillus, green and hemolytic streptococci, etc. These organisms, while in a sense a normal flora, are not so well established as the flora of some other regions, and the nature of the microorganisms present may be determined in large part by the kind of bacteria which are constantly entering the upper respiratory tract.

As long as the resistance of the host is maintained at a sufficiently high level, the bacteria constituting the normal flora do no harm. If, however, resistance is reduced in some manner, the more virulent forms may invade the tissues and set up an infection. The congestion of the nasal mucosa, and the consequent interference with ciliary activity and the movement of mucus, which follows the temperature shock of chilling, not infrequently make possible infection by bacteria such as hemolytic streptococci or pneumococci, which are already present.

[84] Fredericq and Levine: Jour. Bact., 1947, 54:785; Halbert: Jour. Immunol., 1948, 58:153.

THE TRANSMISSION OF INFECTION

Whatever the pathogenic powers of a bacterium and the efficiency of the defensive mechanisms of the host, an essential preliminary to the production of infectious disease is a meeting of the parasite and its prospective host. In some instances in which the bacterium is naturally saprophytic, it enters the body by accident, so to speak; such, for example, appears to be the case in tetanus, gas gangrene and similar infections. In most instances, however, the bacteria that produce disease are more or less closely adapted to a parasitic existence, and pass from one animal body to another with only a relatively brief sojourn in the external world. In general, then, the transmission of infection is a process in which the causative microorganism is transferred, either directly or indirectly, from a diseased to a healthy susceptible animal.

The elucidation of the mechanisms involved in this transfer is a matter of considerable practical as well as theoretical importance. If the sequence of events that precedes infection is known, it may be, and often is, possible to interrupt it at its most vulnerable point and thereby control the spread of disease. From the theoretical point of view, disease is by no means entirely a matter of host resistance and microbic virulence; it is, in a very real sense, the outcome of the interaction of the host and parasite populations. It is at this point that the study of the infectious diseases transcends the bacteriology of clinical medicine with its emphasis on the individual case, and assumes broad biological significance as a problem in interspecies competition.

The equilibrium that tends to become established between the host and parasite populations is an unstable one in that the factors which determine it—*i.e.*, the character of the host population in particular and possibly that of the parasite population, as well as the environmental factors which affect their relationship—are constantly shifting, and the equilibrium ever has a tendency to establish itself at a new level. The shift may be a sudden and violent one whose outward manifestation is an explosive outbreak of disease or, less commonly, may take the form of a gradual increase or decrease in the incidence of the disease.

The factors associated with the maintenance or shift of this equilibrium are the subject matter of *epidemiology*.[1] The term epidemiology is best regarded in this broad sense and therefore includes the study of the transmission of *endemic* disease, *i.e.*, disease which has a low incidence but is con-

[1] For a general discussion of the principles and methods of epidemiology see Frost: *Collected Papers*, pp. 493–542. Commonwealth Fund, New York. 1941. A brief discussion of theoretical epidemiology is given by Aycock and Russell: Amer. Jour. Med. Sci., 1943, 206:399.

stantly present in a population, as well as the study of *epidemic* disease, *i.e.*, disease of high morbidity which is only irregularly present in clinically recognizable form. In this connection it may be noted that the term *pandemic* is often applied to an epidemic of unusually great proportions. These categories, although useful, are not mutually exclusive; a disease endemic in a community may, at times, attain the proportions of an epidemic and later subside to an endemic level.

Essential to knowledge of the epidemiology of disease are certain characteristics of the etiologic agent and of the clinical infection which determine the possible channels of transmission. The more important of these are:

(1) the route by which the infective agent enters the body;
(2) the route by which the infective agent leaves the body;
(3) the resistance of the microorganism to the deleterious effects of the outside environment;
(4) presence or absence of an intermediate host; and
(5) the relation between frank, clinically recognizable disease and the discharge of virulent bacteria from the body.

The Carrier. With regard to the last, additional explanation is desirable. In the early days of bacteriology it was assumed that the contact of host and parasite could have only one or the other of two outcomes; either no infection occurred, owing presumably to high resistance on the part of the host, or clinically characteristic disease developed in the individual. More recently it has become clear that an intermediate state, the establishing of a symptomless infection, may occur. Such infections are, of course, inapparent and concealed and may be demonstrated only by isolation and identification of the infectious agent. An individual so infected is termed a carrier.

Two types of carriers are commonly differentiated, the casual carrier who harbors the microorganism temporarily, a matter of a few days or weeks, and the chronic carrier who remains infected for a relatively long time, sometimes throughout life. Such individuals serve, of course, to disseminate the infectious agent. In the first group are the great majority of carriers of the diphtheria bacillus, meningococcus, pneumococcus, certain streptococci, etc., and the second includes carriers of certain of the enteric bacilli, especially the typhoid bacillus. A third type of carrier is often differentiated, the convalescent carrier who remains infected for a greater or lesser length of time after recovery from the disease. These last do not, of course, fall into the category of concealed infections. Sharp separation is sometimes not possible, however, for the casual or chronic carrier may, in fact, be convalescent from the disease in a form either atypical or so mild as to go unrecognized (ambulatory cases).

While it is now commonplace to recognize the existence of the carrier state, the implications of the general principle that infection may occur without disease are frequently neglected. Thus it follows that clinically apparent infections may constitute only a part, in some instances only a very small part, of the infections continually taking place. Clearly, then, if an important proportion of the infections are of the concealed type, a reasonably accurate estimate of the extent to which the infection is disseminated in the host population cannot be arrived at on the basis of cases of the disease. The implications

of this are several. For example, diseases which occur sporadically and do not seem to be easily or often transmitted from the sick to the well, such as poliomyelitis, meningococcus meningitis, lethargic encephalitis, pneumococcus pneumonia, etc., may be as widely disseminated and readily communicable as measles or the common cold, but the clinically distinctive disease is the exception rather than the rule. Furthermore, the rise or decline of infectious disease or its age or geographical distribution may not reflect a corresponding variability in the prevalence of the infection but may, rather, be a consequence of variation in the case-carrier ratios. Though here inferred from the observed occurrence of the carrier state, none of these possibilities remains purely hypothetical for all have been found to exist. Thus, carriers of virulent pneumococci do not occur predominantly in the higher age groups, nor diphtheria bacillus carriers in the school child, where the morbidity of these diseases is highest; diphtheria bacillus carriers are as common in the tropics as in temperate climates despite the relative rarity of clinical diphtheria in the hot climates, and so on. In many other cases, such as that of poliomyelitis, such a situation is suspected but technically difficult to prove. It will be obvious, therefore, that the recognition of the carrier state and its implications is basic to sound epidemiological thinking and of primary importance to the understanding of the mechanism of spread of the infectious diseases.

The epidemiological factors given above are most readily and most satisfactorily determined by experimental study when a disease is of known etiology, but sometimes may be approximated to a relatively satisfactory degree by indirect evidence. For example, although the famous Broad Street Pump epidemic occurred prior to the discovery of the etiology of cholera, the indirect evidence plainly indicated to Snow that the infective agent left the body in the feces and entered the gastro-intestinal tract via the contaminated well water.[2]

Epidemiological Types of Infectious Disease. On the basis of such fundamental information, the infectious diseases may be separated into a number of *epidemiological types* which, despite certain limitations, serve to illustrate the diversity of ways in which infection may be disseminated.[3] A rough classification, based on the assumption that the human being is the recipient of infection and that the control of diseases of man is the point at issue, follows:

(1) diseases of lower animals transmissible directly to man (rabies, tularemia, glanders, etc.);

(2) diseases of animals or man transmitted by insect vectors in which
    (a) the insect acts as a mechanical carrier (the house fly and typhoid fever),
    (b) the parasite multiplies in the insect vector (bubonic plague),
    (c) the parasite is transmitted from one insect generation to the next by egg infection (spotted fever), and
    (d) the parasite undergoes a portion of its life cycle in the insect (malaria);

(3) diseases of animals or man transmitted indirectly
    (a) by water (the enteric infections such as typhoid fever, cholera, etc.),
    (b) by milk (scarlet fever, bovine tuberculosis, undulant fever, etc.),

[2] The original papers have been reprinted under the title *Snow on Cholera.* Commonwealth Fund, New York. 1936.

[3] Epidemiological types of infectious disease are discussed briefly by Baker: Amer. Jour. Trop. Med., 1943, 23:559.

        (c) by food (typhoid and paratyphoid fevers), and
        (d) by inanimate objects or fomites, such as books, towels, etc. (scarlet fever, diphtheria and the like);
    (4) diseases of man transmitted directly
        (a) by infective droplets—air-borne infection (the respiratory diseases and others) and
        (b) by direct contact (the respiratory and venereal diseases in particular).

Air-Borne Infection. Of these modes of transmission air-borne infection is one of the most important. In recent years Wells[4] and his associates have shed new light on the mechanism of transmission of disease, especially respiratory disease, from man to man by the experimental elucidation of droplet infection. It was postulated many years ago by Pflugge that such diseases could be transmitted by infective droplets. The droplets which he studied, however, were greater than 0.1 mm. in diameter and fell to the ground soon after expulsion by sneezing or coughing, seeding the air for only negligible distances. Epidemiological considerations, however, seemed to demand air-borne infection effective at considerable distances. Wells and his co-workers have shown that Pflugge's evidence was incomplete and that under the usual conditions of humidity particles or droplets smaller than 0.1 mm. in diameter are evaporated completely before reaching the ground, leaving suspended nuclei consisting essentially of organic matter, salts and bacteria, to become, for all practical purposes, a part of the atmosphere. The survival of pathogenic microorganisms in such nuclei is, of course, largely a matter of their resistance to drying, and it has been shown that some of the respiratory pathogens, including viruses such as that of influenza, may remain viable and infective for many hours under these circumstances.[5] The process of expulsion of such droplets in coughing, sneezing and talking has been photographed by Jennison[6] and is illustrated in Fig. 30. Studies of the air in various types of rooms under various conditions have yielded data on the quantitative aspects of air contamination; green streptococci have been used as indicators of air pollution in much the same manner as *Bacterium coli* is used for studies on water pollution. In general, enormous numbers of bacteria of respiratory origin have been found in the air of crowded rooms, especially when the occupants are sneezing, and there can be no doubt of the significance of these observations in relation to the explosive spread of respiratory disease in a non-immune population.

    Evidence has accumulated in recent years[7] which strongly suggests that the inhalation of air-borne bacteria in dust may be an even more important factor in the dissemination of infectious disease of the respiratory tract than that of directly expelled infected droplets. The use of oiled blankets and floors under experimental conditions in hospital wards has, in a number of instances, proved a highly efficacious method of control of the spread of infection. The direct destruction of air-borne bacteria by the use of glycol and

[4] See the review articles by Wells: Jour. Amer. Med. Assn., 1936, 107:1698; and by Wells and Wells: Amer Jour. Med. Sci., 1943, 206:11.
[5] *Cf.* the review by Buchbinder: Jour. Amer. Med. Assn., 1942, 118:718.
[6] Jennison: Amer. Assn. Advancement Sci., Pub. No. 17, 1942, p. 106.
[7] For a discussion see Amer. Jour. Pub. Health, 1948, 38:409.

other bactericidal aerosols (p. 150) and ultraviolet irradiation is not only feasible in some circumstances but has given encouraging results.

Fig. 30. The atomization of mouth and nose secretions demonstrated by high speed photography. *1*, A violent sneeze in a normal subject; note the close approximation of the teeth, resulting in effective atomization. *2*, Head cold sneeze; note the strings of mucus and the less effective atomization of the viscous secretions. *3*, A stifled sneeze. *4*, Sneeze through a dense face mask. *5*, Cough; note the lesser discharge than in the uninhibited sneeze. *6*, Enunciation of the letter f. (Jennison.)

Even though air-borne infection, whether by infected droplets or dust-borne bacteria, is an important factor in the spread of respiratory disease, very often direct contact with infected persons may assume a major role.

As may be inferred from the outline above, a disease not infrequently has

a certain epidemiological character which may at times be even more familiar to the epidemiologist than the causative agent is to the laboratory worker. In the case of epidemic influenza, for example, the disease is, at present, an epidemiological rather than clinical entity and is diagnosed with reasonable accuracy only during epidemic periods. Similarly, epidemiological studies have shown the existence of two kinds of smallpox, the one a relatively innocuous variety with a low case fatality and the other a severe disease (so-called malignant or "black" smallpox) with a high case fatality. The difference between these varieties is real and of no small practical importance even though indistinguishable by clinical observation of the individual case (p. 859).

The epidemiology characteristic of a given disease shows, on the one hand, certain, and sometimes close, relationships to other similar infections, and, on the other, a certain variability which arises as a result of transmission in more than one way. The enteric infections, cholera, typhoid fever and the bacillary dysenteries, are similar to one another in epidemiology but are quite different in this respect from the respiratory diseases or the insect-borne diseases. The epidemiology of typhoid fever, however, is variable within limits and depends to some degree upon whether the disease is water-borne, milk-borne or transmitted by food or contact. Clearly, then, although it is possible to speak of certain broad principles of epidemiology, the epidemiology of a disease is in itself a special case and must necessarily be considered elsewhere (see later chapters) under the head of that disease.

The epidemiological character of a disease is dependent primarily upon certain aspects of the microorganism and the clinical infection as indicated above, and secondarily upon the habits, environment and mass susceptibility of the host population. To return to the example of typhoid fever: any mode of transmission is necessarily one that provides a connecting link between infectious fecal matter and the mouth of a susceptible human being, and the possibilities are, to this extent, limited. The age, sex and seasonal incidence, to some extent the case fatality, and the geographical and sociological distribution of cases are reflections of both the mode of transmission and the character of the population. In the case of water-borne typhoid, the drinking water supplies the connecting link, and the ensuing epidemic, limited to the area supplied by the contaminated water, shows no respect for age, sex or economic status. Milk-borne typhoid, on the other hand, geographically limited by the route by which the infected milk is delivered, exhibits an increased incidence in the lower age groups and among females, and is somewhat more frequent among those of higher economic status. Other modes of transmission are similarly reflected as minor variations in the epidemiological character of the disease.

Although the characteristics of the infectious agent and the clinical disease determine the *means* by which disease may be transmitted, the *extent* of its spread is a mass phenomenon determined by the character of the host and parasite populations and, as a corollary, their interaction with one another.

The Bacterial Population. The well-known variation in the severity and "contagiousness" of the infectious diseases is a consequence of corresponding variation from one species of pathogenic bacteria to another in

their ability to invade the body tissues and, once established, to produce clinical disease. As indicated in a previous chapter, a single species is potentially variable in these respects, for such variation can be induced by appropriate experimental manipulation. The possibility of such intraspecies variation in a bacterial population existing under natural conditions is one that has intrigued students of infectious disease for many years.

It is tempting to account for the genesis and rise of epidemic disease by assuming that the causative agent of a disease of endemic proportions gains in virulence by successive passage from person to person until its pathogenicity is so enhanced that an epidemic ensues. Similarly, a sojourn in a host population containing an increasingly large proportion of immunes might be expected to result in a diminution in the virulence of the microorganism and consequent subsidence of the epidemic. Furthermore, successive epidemic waves might conceivably result from periodic fluctuations in the virulence of the parasite. Unfortunately for such an explanation, there is little or no direct evidence that alterations in virulence play an important part in the evolution of single or secondary epidemic waves, and, in nature, bacterial virulence appears to be a relatively stable character.[8]

On the other hand, differences in the severity of a single disease from one epidemic to another are, in part, attributable to the existence of strains of the infectious agent which differ from one another in virulence. Benign and malignant smallpox referred to above is a case in point, and some workers believe that some strains of the diphtheria bacillus produce a more severe disease, (*i.e.*, with a higher case fatality) than others (p. 611). Although variable from one strain to another, available evidence indicates that within a single strain virulence does not fluctuate to a demonstrable degree.

Possibly attributable in part to alterations in bacterial virulence are the changes in morbidity and mortality of some diseases such as scarlet fever, syphilis and tuberculosis, over long periods of time. In the case of scarlet fever the twenty-five years prior to 1830 was a period of very low death rates and was followed by a forty-year period of high death rates. Since then the death rate has declined and, though the incidence remains high, the case fatality is relatively low. In other diseases only a decline has been observed. Syphilis is no longer the scourge it was in the sixteenth century, and the present decline in tuberculosis began before the institution of preventive and therapeutic measures. In still other diseases, such as measles, no such long-term alterations in prevalence have been observed. Information is as yet too limited to assess these phenomena; possibly in some diseases there are long-term periodic fluctuations in bacterial virulence (this may be an artifact and represent only variations in the prevalence of virulent "epidemic" strains of the parasite) while in others an adaptive reduction in virulence or increase in resistance on the part of the host or a combination of both may play a part.

In general, it may be said that, in the short view, the bacterial population, as it exists in nature, is remarkably stable in so far as its ability to produce disease in a host population is concerned. Although the severity of a disease

[8] *Cf.* Aycock, Lutman and Foley: Amer. Jour. Med. Sci., 1945, 209:395.

may, and often does, vary from one epidemic to another, variation in virulence is not an important factor in the single epidemic wave. Over long periods of time, however, alterations in virulence may contribute to the changes in morbidity and mortality observed in some of the infectious diseases.

The Host Population. In contrast to the relative stability of the bacterial population, the human population is highly variable in its resistance to infection, and the variation, attributable to both intrinsic and extrinsic factors, is not infrequently of such magnitude that its consequences are of considerable practical importance.

Since a population, human or infrahuman, is composed of individual organisms, it follows that its character is determined by the nature of these individuals and their relations to one another, and its reaction to an external influence is expressed in terms of the aggregate of the reactions of its members. The response of a human population to an infectious disease is, of necessity, measured in terms that are composites of the responses of the individual members of the population—in short, by some method of counting. Such counting is, of course, the basis of statistics, and the statistical method, with its ramifications and refinements, is a powerful tool which makes possible the study of the response of human populations to disease—a response which is measured in terms of rates, ratios, life tables and similar numerical devices.

Of the intrinsic factors which determine the response of a human population to an infectious disease, one of the most important is age distribution. The quantitative predominance of the lower age groups characterizing an immature population declines with population growth while the higher age groups correspondingly increase. As a consequence, the diseases of childhood and early adult life, such as diphtheria, tuberculosis and the like, are relatively prevalent in an immature population but become progressively less so with the passage of time, while the diseases of old age increase in incidence with the maturing of the population.[9] The frequency of an infection is expressed as a rate, either as the number of persons infected in a given unit of time or *incidence*, or as the total number of persons infected at any one time or *prevalence*. Correction of morbidity and mortality rates is a practical necessity and is made either by the use of *specific rates, i.e.,* the proportion of cases or deaths within a specified age group, or by the use of *standardized rates* which are not the observed rates but rather what the observed rates would be if the age distribution of the population were that of a standard or reference population.[10]

The sex distribution of a population and its racial composition are of somewhat lesser practical significance, although in some communities in the

---

[9] For a discussion of these problems see Perrott and Holland: Milbank Mem. Fund Quart., 1940, *18*:359.

[10] Two standards have been used in Europe, one, the population of Sweden as it existed in 1890, and the other, the population of England and Wales as shown by the 1901 census. The first is standard in age distribution only, while the second is standard for both age and sex. In this country the population of the entire country is usually taken as a standard, and the populations of states or other portions of the country compared or adjusted to it.

United States the high mortality rate of the Negro has necessitated the use
of race-specific mortality rates.[11]

The extrinsic factors which alter the resistance of a host population to the
spread of disease may exert their effects in either or both of two ways: first,
by influencing the resistance of part or all of the individuals comprising the
population; and, second, by influencing the relationships between individuals.
Perhaps the most important factor in the first category is active individual
immunity. If a sufficient portion of a population is immune to a disease as a
result of artificial inoculation or recovery from an attack, the resistance of the
entire group to epidemics of that disease is of a high order, a phenomenon
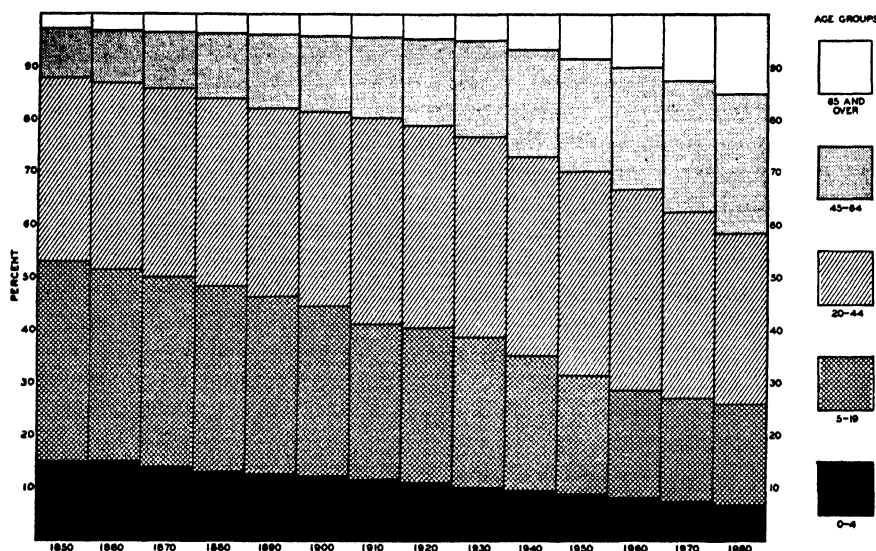
Fig. 31. Changing age distribution in the population of the United States. Estimates 1940
to 1980 by Thompson and Whelpton.

which has been termed *herd immunity*. Other factors may reduce the resist-
ance of the individual; in times of stress or calamity, for example, when rela-
tively large groups are undernourished, fatigued and exposed to inclement
weather, epidemic disease may spread with great rapidity.

Equally important to the resistance of a population to epidemic disease
are the factors which determine the interrelationships of its members. Crowd-
ing in large gatherings or the enforced close association arising from inade-
quate housing facilities obviously provides opportunity for the dissemination
of respiratory and other diseases transmitted directly from man to man, and,
as well, certain indirectly transmitted infections, such as louse-borne typhus
fever. Similarly, the spread of enteric infections is, to a large extent, depend-
ent upon sanitary facilities and the solution of the twin problems of water
supply and sewage disposal. Group practices which support a large rat popu-

[11] For a discussion of the methods of population study with respect to disease see Pearl:
Introduction to Medical Biometry and Statistics, 3rd ed. W. B. Saunders Company,
Philadelphia. 1940.

lation make bubonic plague a potential menace, and the presence of large numbers of mosquitoes of the appropriate species allows the wide dissemination of malaria and yellow fever. These and other factors, political, sociological or economic in nature, obviously exert no small influence on the resistance of a population to the spread of disease.[12]

The Interaction of Host and Parasite Populations. It will be clear from the foregoing discussion that the interaction of the host and parasite populations is a highly complex phenomenon. Even assuming that the parasite population, when the parasite is a pathogenic bacterium, remains relatively constant in its ability to produce disease, the resistance of the host population is in a constant state of flux and the equilibrium between the two is rarely a "steady state." As has been indicated, the relation between host and parasite populations is a part of the general problem of interspecies competition, and has been studied at length, particularly by the economic entomologist[13] and the experimental zoologist.[14] The infectious diseases of man constitute a series of special cases of the host-parasite relationship, differing from one another with respect to mode of transmission, incubation period, period of infectivity, immunity, case fatality, etc. The studies on infectious disease have taken two forms: one, the theoretical analysis of epidemic spread; and the other, the experimental investigation of controlled epidemics among populations of laboratory animals, *i.e.*, experimental epidemiology.

Theoretical Analysis. The theoretical treatment of the dissemination of infectious disease in a susceptible population is exceedingly difficult because of the tremendous number of variables which are involved. If, however, certain simplifying assumptions be made, analysis in terms of the mathematics of probability yields information of considerable significance, and a certain insight into the evolution of the single epidemic wave may be gained.

The evoluton of the single epidemic wave is best considered here by the arithmetic method of Frost,[15] which involves finite differences. Let $C =$ the number of cases, $S =$ the number of susceptibles and $N =$ the contacts per day, then r, the contact rate per day, is given by

$$N = rCS \text{ or } r = \frac{N}{CS}$$

It is assumed that each case is infectious, that one contact suffices to produce the disease in a susceptible individual, that multiple contacts are disregarded, and that the unit of time (the day) is small enough so that C and S do not change materially during that period. Then the number of contacts per unit time t (the day) is

$$Nt = rCSt$$

The probability of contact is, therefore,

$$p = \frac{Nt}{S} = rCt$$

---

[12] Sigerist: *Civilization and Disease.* Cornell University Press, Ithaca. 1943.

[13] See the reviews by Nicholson: Jour. Animal Ecol., 1933, 2:132; and by Thompson: Parasitology, 1939, 31:299.

[14] Gause: *The Struggle for Existence.* Williams & Wilkins Company, Baltimore, 1934.

[15] Frost: Cutter Lecture, 1928, unpublished. Method outlined by Zinsser and Wilson: Jour. Prev. Med., 1932, 6:497.

and the probability of avoiding contact is

$$q = 1 - p = 1 - rCt$$

Since there are $1/t$ units of time in the entire period, the chance of avoiding contact over this period is

$$Q = (1 - rCt)^{\bar{t}} = e^{-rC}$$

Therefore, the chance of at least one contact is given by

$$P = 1 - e^{-rC}$$

and the number of new cases infected during the day is

$$PS = (1 - e^{-rC})\, S$$

Assuming the incubation period to be one day—*i.e.*, the contact of one day is the case of the next—a theoretical epidemic wave may be built up by a series of substitutions in the last equation. Starting with 10,000 susceptibles, one case, and a contract rate, r, of .0002, the first day,

$$(1 - e^{-.0002})\ 10,000 = 2 \text{ (new cases).}$$

the second day,

$$(1 - e^{-.0006})\ 9998 = 6$$

the third day,

$$(1 - e^{-.0018})\ 9992 = 18$$

and so on through the complete epidemic wave. Various modifications such as limiting the period of infectivity through the introduction of case fatality and the development of immunity, extending the incubation period, and the like may, of course, be made. This kind of treatment is, of course, an application of the law of mass action. The first relationship, $N = rCS$, becomes $rCS = dC/dt$ and may be manipulated in a variety of ways. Wilson[16] has developed this approach at some length.

Theoretical epidemics built up in this way show a remarkable similarity to observed epidemics of disease, and, although the factors entering into the determination of the value of r are highly complex, it is evident that the probability of chance contact is a factor of primary importance in the evolution of the epidemic wave. In a population consisting largely or entirely of susceptibles, this probability is large and the disease spreads rapidly; but, as the number of susceptibles is reduced by conversion to cases, immunes and fatalities, the probability diminishes and the epidemic subsides.[17] It may be noted that the term "contact" is used to mean "effective contact" and more than one meeting with a case may be required to make up an effective contact. It is often said that an important factor in epidemic spread is dosage, *i.e.*, the number of microorganisms that a susceptible individual encounters within a definite period of time. An adequate "dose" is, clearly, an effective contact.

[16] Wilson and Worcester: Proc. Nat. Acad. Sci., 1944, 30:37, 264; *ibid.*, 1945, 31:24, 142, 203, 327.

[17] For experimental studies on the mathematical theory of epidemics see Kermack and McKendrick: Jour. Hyg., 1939, 39:291 *et ante*. Also the summary by McKendrick: Edinburgh Med. Jour., 1940, 47:117.

The nature of herd immunity will become clear at this point, for the higher the proportion of immunes in a population the smaller the probability of effective contact between case and susceptible; *i.e.*, many of the contacts will be with immunes, and the population exhibits a group resistance to epidemic disease which may be of such a high order that an epidemic is no longer possible and the disease smolders in an endemic form as a result of the importation of new cases or the persistence of infection in healthy carriers whose contacts will give rise to an occasional case. A susceptible member of such an immune population, then, enjoys an immunity that is not of his own making but arises as a result of his membership in the group. A measure of this protection is given as Q in the above equations.
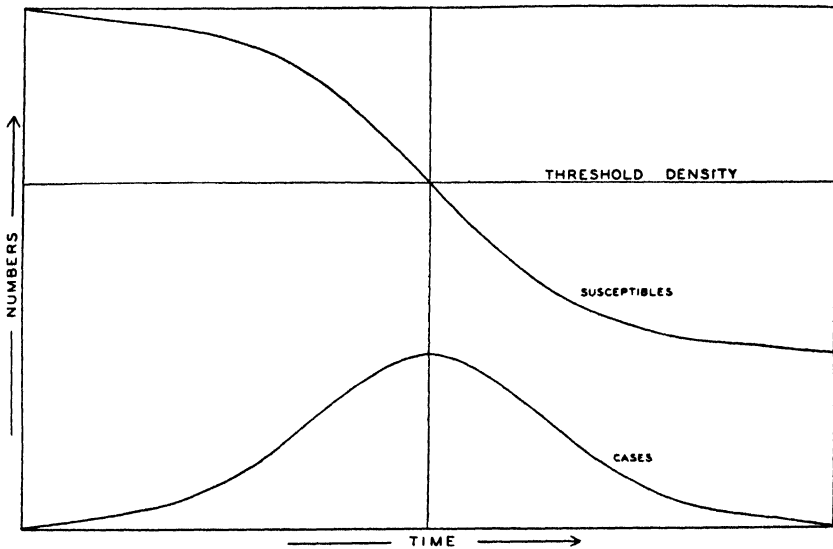
Fig. 32. Diagrammatic representation of the course of an epidemic wave in terms of numbers of cases (lower curve) and numbers of susceptibles (upper curve). Note the coincidence of the peak of the epidemic wave with the threshold density of susceptibles. (After McKendrick.)

Clearly there will be a critical proportion of susceptibles and immunes in the population; with a greater number of susceptibles epidemic disease can develop and with a lesser number it cannot. This critical point has been termed *threshold density* and is that which prevails at the peak of the epidemic wave. The total number of cases in the epidemic will, then, be twice the number of susceptibles in excess of this threshold density present at the beginning, and following subsidence of the wave the population is left with a greater or lesser degree of herd immunity.

Such precise relationships are only approximated in nature. In practice the threshold density fluctuates and is a function in part of dosage. When the prevalence of a disease is high a given individual will be subjected to a greater number of bacteria per unit time; some, previously immune to smaller doses, will become susceptibles under such circumstances and the

"effective concentration," so to speak, of immunes declines with consequent effect on the herd immunity to epidemic disease. This effect of dosage on the host population has been termed *infection pressure* and is a matter of considerable practical importance. Successive epidemic waves may, then, occur as a consequence of increased infection pressure as well as through the accumulation of new susceptibles (see below). Recurrent waves of a disease that is a clinical but not an etiological entity are, of course, another matter.

The question of the relationship between an infectious disease and a susceptible population over a long period of time is somewhat more complex. Mathematical treatment such as the Ross malaria equations and Martini's equations for immunizing diseases[18] is too far removed from reality to have much practical significance, principally because the mathematical approach requires that the parameters remain constant over long periods of time. This condition is probably rarely if ever satisfied; it is well known, for example, that within the last century both diphtheria and scarlet fever, previously of low prevalence, took on a malignant character and higher prevalence for some decades and then within the last forty years have gradually declined in both prevalence and severity. It is of some interest, however, that the differential equations of Martini, for example, show one stable equilibrium at the origin and another at a positive value which is approached by a series of oscillations above and below the final state of equilibrium so that a series of epidemic waves appears. These solutions suggest that (a) under certain circumstances a disease may tend to die out and (b) under other circumstances a disease will reach an equilibrium after a series of epidemics of decreasing severity.[19] Such periodicity in the incidence of many infectious diseases is well known; one of the best examples is that of measles which recurs in epidemic form at approximately two-year intervals. So far as is known, however, no infectious disease occurring under natural conditions shows a damped periodicity approaching invariancy though it has been found to occur in experimental epidemics in which the immigration rate of the new susceptibles is very high (*vide infra*).

A disease will, of course, die out if it does not reproduce itself, *i.e.*, if the bacterium is present only in the active case and each case does not, on the average, give rise to a second case. It is not unlikely that some diseases are dying out in this way; it has been suggested,[20] for example, that the decline in tuberculosis is due in part to inability of the disease to reproduce itself, a process accelerated by isolation of active cases. The second suggestion is of interest in connection with the epidemic periodicity of certain diseases such as measles, which appears in epidemic form usually at two year intervals. It is obvious, of course, that following an epidemic a new crop of susceptibles appears, and when their numbers reach a sufficiently high level, a new epidemic ensues and so on *ad infinitum*. It is unlikely, however, that these epidemic waves are damped as predicted by theoretical equations, for subtle variation in the complex of factors that are oversimplified as parameters

[18] For a discussion of this general problem see Lotka: *Elements of Physical Biology.* Williams & Wilkins Company, Baltimore. 1925.

[19] See Wilson and Worcester: Proc. Nat. Acad. Sci., 1945, *31*:294.

[20] Frost: Amer. Rev. Tuberc., 1935, *32*:644.

will wipe out the damping effect. It will probably be many years before predictions regarding the future of infectious diseases attain a status better than that of guesses.

Experimental Epidemiology.[21] The information that may be derived from observation of naturally occurring disease, *i.e.*, descriptive epidemiology, is limited since the observer has no control over the process and for all practical purposes the experiment is carried out for instead of by him. In experimental epidemics, however, the conditions may be adjusted as desired and it may be anticipated that such experiments will be highly informative.

In recent years the study of epidemics of infectious disease developed in populations of laboratory animals under controlled conditions has been carried out by Topley, Greenwood, Wilson and others in England and by Webster and his colleagues in the United States.[22] These experimental epidemiological studies have been confined, for the most part, to the study of the dissemination of *Salmonella typhi-murium* (*aertrycke*), *Salmonella enteritidis, Pasteurella muriseptica* and the virus of ectromelia (a foot-pad disease of mice) among populations of mice. Mouse typhoid and pasteurellosis are regarded as analogous to human diseases such as typhoid fever in which there is an imperfect immunity and carrier state, and ectromelia as analogous to human diseases in which a solid immunity is developed, such as diphtheria. The extent to which the analogies may be carried, and to which the conclusions reached are applicable to the considerably more complex human population as it exists under natural conditions, is open to some question; nevertheless, these studies, as yet in their infancy, have yielded valuable information.

The experiments which have been carried out were of two general types. The closed epidemic was produced in a population of mice, often about fifty in number, by the introduction of infected animals. The other type of experiment was carried out in an infected mouse population recruited by continuous immigration, *i.e.*, mice were added at regular intervals, the rate varying from one mouse every three days to six mice per day. The results may be summarized briefly:

(1) The epidemic wave initiated in the closed population by the introduction of infected individuals closely resembled those observed in the human population.

(2) The effects of dispersal of the infected population of the closed epidemic into large or small groups at various times during the development of the epidemic were studied. The time at which the population was dispersed was found to be of primary importance; the later dispersal was effected, the less favorable the result, and after the peak of the epidemic wave was reached it continued unchecked even though the population was dispersed to individual mice. This is of particular interest since fleeing from an epidemic has been a popular method of escaping infection, *viz.* the *Decameron.* In this connection the results of dispersal of children from the industrial centers of England in 1939–1940 may be noted. A sharp reduction of 40 per cent or more in diphtheria morbidity occurred in the evacu-

---

[21] See the general discussion by Topley: Proc. Roy. Soc., Ser. B, 1942, 130:337.
[22] See the review by Greenwood, Hill, Topley and Wilson: Medical Research Council Special Report Series No. 209. 1936; Webster: Medicine, 1946, 25:77.

ated towns together with an increase of 60 to 70 per cent among local children in reception centers, the latter returning to normal within six months. Similarly, the biennial periodicity of measles was broken by this dispersal.[23]

(3) The effect of active immunization on the epidemic was not so great as might be supposed. If the mice were immunized prior to the epidemic, a favorable effect was apparent and more marked in ectromelia than in *S. typhi-murium* infections. Immunization undertaken after the initiation of the epidemic was without effect.

(4) In the mouse population recruited by continuous immigration a series of epidemic waves developed, the frequency of which was directly related to the rate of immigration. Thus, with a low rate, such as one mouse every three days, epidemic waves were separated by periods of remission in which no deaths occurred. As the immigration rate was increased, the waves occurred with greater frequency and with the addition of six mice per day there were no periods of complete remission, the epidemic waves taking the form of periods of increased mortality.

(5) With high immigration rates, the peaks and troughs in the daily death rate became less and less pronounced after a year or more, both the rate and total population tending to become invariant. Thus it was possible to approximate experimentally the damped periodicity and eventual equilibrium predicted on theoretical mathematical grounds (see above), and the experimental host population could be brought into approximate equilibrium with the parasite population.

(6) An inadvertent experiment with a population recruited by a high rate of immigration proved to be of considerable interest. The mouse population had reached the equilibrium indicated above by summer when there was a heat wave of unusual intensity in London. There were many non-specific deaths (mice are highly susceptible to heat), followed by violently fluctuating specific mortality. In the course of time an equilibrium was again reached but at a considerably higher death rate and decreased numbers than formerly. The parasite was shown to be unaltered in virulence and the immigrants had not, of course, had the heat experience. Hence the host-parasite relationship *per se* was altered by an environmental "catastrophe." The relationship of these observations to the long term relation between the human population and infectious disease is of interest. If it be assumed that the day of the mouse is equivalent to thirty days in the life of man, over the four years taken by this experiment the following were observed: (a) a period of stability with low regular death rates and growing population (*ca.* 600 days) equivalent to a human experience of slightly less than fifty years —this, it may be noted, is longer than the twenty-five year period ending about 1830 during which the death rate from scarlet fever was relatively low; (b) a period equivalent to fifteen years of severe and repeated epidemic waves; and (c) a period equivalent to some thirty years of relatively high mortality in a more or less stable state of equilibrium in a population reduced in numbers—it may be noted that the post-1830 epoch of virulent scarlet fever lasted more than forty years.

(7) The mortality rates of the mice were intimately related to the length

[23] Stocks: Jour. Roy. Statistical Soc., 1941, *104*:311; *ibid.*, 1942, *105*:259.

of time that they were members of the infected population; the rate rose rapidly to a peak in the early days of cage age and, although it declined slowly with the passage of time, to a greater extent in ectromelia than in mouse typhoid or pasteurellosis, the survivors of one epidemic wave may be the victims of another. The removal at regular intervals of a number of mice equal to the number added at the same intervals markedly altered the trend of these mortality rates; under these circumstances, the initial peak of mortality was lower, but the rapid decline was not apparent and the mortality rate remained at a high level.[24] Removal was of considerable advantage to the individual mouse; in general, the earlier the removal the greater the advantage, except during the decline of an epidemic wave, when isolation did not increase the chances for survival of individual animals.

Although the evolution of the single epidemic wave in the experimental studies closely resembles its counterpart in the human population, the inability to control experimental epidemics by immunization is at variance with observations on epidemic disease in human populations. Some human diseases, such as diphtheria and smallpox, can be restrained from assuming epidemic proportions by active immunization of a sufficiently large number of the individuals making up the population. Whether or not this and certain other minor discrepancies prove to be real or illusory with future work, a number of pertinent suggestions have come out of these investigations. The response of the experimental host population in terms of mortality rates and cage life expectation cannot be interpreted as yet in some cases; in others the interpretation is questionable. For example, the English workers feel that some of the data suggest alterations in virulence and "infectivity" of the bacteria, while Webster in the United States interprets these findings as indicative of a graded, inheritable resistance in the host population.

Some of the experiments, however, have yielded clear-cut results that bear directly upon the interaction of host and parasite populations in nature. Perhaps the most important of these is the experimental demonstration of recurring epidemic waves resulting from the continuous addition of new susceptibles to the infected population. In spite of the fact that such a sequence of events is predicted by theoretical epidemiology, as indicated above, the repeated flaring up of a disease of man or domestic animals, thought to be stamped out in the inter-epidemic periods, is most often taken as evidence of the reimportation of infection.[25] The experimental demonstration of this

---

[24] Greenwood, Hill, Topley and Wilson: Jour. Hyg., 1939, 39:109.

[25] In this connection Major Greenwood (*Epidemics and Crowd-Diseases*. The Macmillan Company, New York. 1935) has made the following delightful suggestion: " . . . a majority of the officials who in different countries report upon epidemics among farm animals cannot bring themselves to conceive that upon a farm or within a district wherein a scheduled disease once existed, and was afterwards officially declared to have ceased to exist, it could start into life without a reimportation of *materies morbi*, these happenings must be explained by reimportation. We can always see a joke much more easily when it is against some poor foreigners rather than against ourselves. I advise an Englishman, therefore, to read the Dutch or German explanations of the recrudescence of foot-and-mouth disease and learn how the virus may be blown by the winds of heaven, or dropped on an island in the excreta of wild geese. A Dutchman might find explanations of the reimportation of foot-and-mouth disease to be found in English official documents equally bizarre."

phenomenon and the indicated futility of quarantine in the control of a disease which has become widely disseminated (only when a disease is not present, or present in limited effective foci, can quarantine and isolation procedures be effective) is, clearly, of considerable significance.

Epidemiological Data and Their Interpretation. The purposes of epidemiological study are threefold: first, to indicate the nature of the infective agent, its source and modes of transmission when these are not fully established otherwise, *i.e.*, on an experimental basis; second, to extend this information into a corresponding general theory of the epidemiology of the disease; and third, to determine in detail the local conditions which favor or control the dissemination of the infection in a given area or community.

For these purposes four general types of information, usually simple in character but wide in extent, are required. First, the *area* in which the disease occurs and the regularity of its geographical distributions are highly informative. Thus, the general occurrence of a disease indicates that environmental conditions, including fauna, climate, etc., peculiar only to parts of the area are not essential to its transmission. Similarly, restriction of the disease within geographic limits indicates that special environmental conditions are necessary for its dissemination; these may include crowding, presence of an insect vector, water supplies, proximity to reservoirs of infection, etc. In general, a uniform distribution of the disease is indicative of a simple method of transmission, as in the case of measles, whereas an irregular distribution, such as that of spotted fever, implies a more complex process dependent upon a source of infection or conditions necessary to transmission that are correspondingly irregularly distributed.

Second, the *rate of prevalence* of the disease suggests the source of infection. A high rate, such as that of measles, indicates that the observed cases are the most important, if not the only source of infection. Conversely, sporadic distribution of widely separated cases implies the existence of a concealed reservoir of infection such as the casual or chronic carrier or some of the lower animals.

Third, the *seasonal distribution* is also informative when considered together with other epidemiological features of the disease. Thus, a seasonal occurrence may be consistent with hypothecated insect transmission or it may eliminate an insect vector as the sole transmitting agent. Similarly, the occurrence of water-borne typhoid fever in the late winter and early spring supports the hypothesis of preservation of typhoid bacilli in the cold and their liberation from contaminated watersheds in the first thaws.

Lastly, the *age distribution* of the disease is suggestive as a corollary to other epidemiological characteristics, and often aids in their interpretation. Thus, a disease occurring for the most part in the early years of life, such as measles or diphtheria, often shows differences in age incidence between urban and rural areas which are explained by the more frequent occurrence of immunizing infections, apparent or subclinical, in the more crowded areas. Since similar differentials are apparent in poliomyelitis, the age distribution of this disease, together with other of its epidemiological features, supports the view that this disease is widely prevalent as a subclinical infection.

Epidemiological evidence, therefore, consists of a series of inter-related facts from which a conclusion or series of conclusions may be drawn. The first step in epidemiological procedure is necessarily the demonstration of associations between the frequency of occurrence of the disease and some conditions or set of conditions, the second the ascertaining of the relationship of these associations with one another, and third their relations to the general epidemiological theory of the disease. To take a simple example, in milk-borne typhoid fever it may be ascertained that cases of the disease occur pre-dominantly along the route of the milkman, that in families so supplied the cases occur among those who drink milk, that the cases appeared within a limited time to suggest simultaneous infection and that an employee of the dairy is a carrier of typhoid bacilli. Not only are these associations related to one another but they are consistent with the general epidemiological theory of typhoid fever, *viz.*, that transmission is basically a matter of the transfer of infected fecal material (or urine) from a case or carrier to the mouth of a susceptible person.

While this would appear self-evident, there is an opinion of some preva-lence that since epidemiological evidence is necessarily purely circumstantial, it cannot be conclusive. This is perhaps due in large part to failure to appre-ciate the development, often basically statistical and therefore mathematical, of the logical analysis, and the significance of the body of evidence as a whole. The method of analysis and interpretation of epidemiological evi-dence is identical with that of experimental evidence, the practical difference lying in the fact that, as pointed out earlier, the epidemic is an experiment done for rather than by the observer and cannot be manipulated.

The Control of Infectious Disease. With an understanding, albeit admittedly imperfect, of the factors which determine the means and extent of the dissemination of infectious disease, comes the possibility of control. The variety of epidemiological types of disease and the individual variability within these broad groups make for differences in control measures, and the control of a given disease under given circumstances almost always consti-tutes a special case. Certain generalizations may, however, be made which will serve to indicate the complex nature of the problems of the control of the spread of infection and provide a point of departure for consideration, in later chapters, of specific diseases.

In general, control measures may be regarded as corollaries of the factors involved in the transmission of infection and, therefore, fall into two groups: one including those which raise the resistance of the individual and, through him, of the population; and the other, those designed to alter the relation-ships of the individual members of the population to one another in such a way that the opportunities for the dissemination of the infectious agent are reduced. Very often both types of control measures are used, their relative efficiency and practicality varying from one disease to another.

Diseases of lower animals which are transmitted either directly or indi-rectly to man involve a third population, the animal reservoir of infection. When transmission is from animal to man and occurs only rarely from man to man, three methods of control are possible. First, the disease may be controlled in the animal; second, the relationship between the human and

animal populations may be altered in such a way that transmission of infection cannot take place; and third, the resistance of the human population may be raised by immunization procedures. The relative efficacy of these methods of control is variable and depends upon the disease. Rabies, for example, can be controlled in the dog population, and, in fact, has been entirely eliminated in England by rigid regulation, but neither mass immunization of the human population nor alteration of the relationship between dog and human populations is practical. In the case of bovine tuberculosis and undulant fever, however, disease may not only be controlled in the animal population, but the relation between human and animal populations is readily altered by interposing the barrier of pasteurization. In other instances in which the reservoir of infection is a wild animal, immunization, when a solid immunity can be obtained, of the limited number of individuals who have contact with such infected populations is satisfactory.

The insect-borne diseases present still other problems in control in that the link between man and animal or man and man is a living organism. In general, control of the insect population or of the disease in the insect will result in control of the disease in man. The infections transmitted by insects are, for the most part, rickettsial, protozoan and virus diseases; few bacterial diseases are insect-borne, presumably because in many of the blood-sucking insects the intestinal tract is actively bactericidal and most species of bacteria are rapidly destroyed there. The mechanical transmission of bacterial infection by insects is, then, relatively rare and, except under unusual circumstances, of no great importance. Two species of pathogenic bacteria are, however, resistant to this bactericidal activity: the bacilli of plague and tularemia multiply in the rat flea (*Xenopsylla cheopis*) and the deer fly (*Chrysops discalis*) respectively. When the microorganism either multiplies or completes a portion of its life cycle in the insect vector, the insect is infective for the duration of its life; such is the case in yellow fever, typhus fever, malaria and other diseases. The problems of control of these diseases are the problems of the control of the insect population, either indirectly through the insect's animal host as in bubonic plague or directly as in malaria. A further complication is introduced in the case of spotted fever. The tick (*Dermocentor andersoni*) undergoes an incomplete metamorphosis, and the infection is "hereditary," *i.e.*, transmitted to the second generation. There is, in consequence, not only an animal reservoir of infection but also a second reservoir in the form of infected ticks.

Diseases Transmitted Directly from Man to Man. The diseases transmitted directly from man to man by contact or by air-borne infection are by far the most difficult to control. The mechanism of transmission is, of course, very simple and does not offer the possibilities for interference that may be taken advantage of in the more complex processes. Furthermore, the practices and habits of the human population that make possible transmission by these means are a part of day-to-day existence in the life of man. Thus the aggregation of human beings under crowded living conditions in urban areas, and the theatres, schools, churches, and other public meeting places cannot be dispersed or eliminated for the control of air-borne infection. Similarly, pre-

vention of the spread of venereal disease is theoretically possible but relatively unsuccessful in practice.

In the past, therefore, control of disease spread by these means has been dependent upon effective active immunization procedures. Diphtheria and scarlet fever may be so controlled by the immunization of a reasonably large part of the population, but influenza, the common cold and others continue to be widespread.

With the elucidation of the mechanisms of air-borne infection and the development of aerosols (p. 150) and ultraviolet irradiation for the destruction of air-borne bacteria, it seems possible that the spread of air-borne infection may be controlled. Thus, the establishing of air sterilization in public gathering places of various kinds, possibly in the air conditioning systems, as well as in hospital wards may be effective.

The control of the spread of a given disease is, in practice, complicated by two important factors. First, a disease is often transmitted in more than one way. This variation may be within narrow limits; for example, an insect-borne disease may be transmitted by several species of insects which differ from one another in distribution, breeding habits and the like. In extreme cases a disease may fall into more than one epidemiological type; bubonic plague, for instance, which is transmitted from the rat to man by the rat flea, may assume a pneumonic form transmitted by infective droplets and become independent of the rat population. Secondly, infection may be carried and transmitted, not only by clinically recognizable cases of disease but also by cases before clinical symptoms appear; by individuals who have a disease in such a mild form that it is not recognized; and by healthy individuals who carry the infection either transiently or semipermanently. For example, while it is not difficult to prevent epidemics of water-borne typhoid fever, the disease remains endemic in a community as a result of the dissemination of the bacilli from ambulatory cases and carriers, and this so-called "residual typhoid" is extremely difficult, if not impossible, to eradicate.

It will be clear from the foregoing discussion that the transmission of infection is indeed a highly complex matter. Each disease is a special case of the host-parasite relationship which is not infrequently complicated by the interaction of two or more host populations. The elucidation of the inter-relationships of these groups is a necessary preliminary to the understanding of the mechanisms operative in the dissemination of disease.

Chapter 10

THE BACTERIOLOGY OF WATER AND SEWAGE

Of the interrelationships between the members of human populations that facilitate the transmission of infection, those arising as a consequence of common water supplies and the group disposal of sewage are among the most important. Transmission of disease by this means is, of course, confined to diseases of the so-called enteric group, in which infection takes place via the gastro-intestinal tract and the causative microorganisms are discharged with the feces. Infection is, therefore, the result of a direct connection between infectious fecal material and the mouth of a susceptible person, and when that connecting link is a common water supply, as it frequently is, outbreaks of cholera, typhoid fever and the like occur. This link is, however, readily broken; the great water-borne epidemics are rapidly becoming a thing of the past as a consequence of the utilization of effective control measures, and present day water-borne epidemics are indicative, not of a lack, but of a failure to make use of existing knowledge.[1]

WATER[2]

Because of water-borne disease and the obvious desirability of its control, studies of the bacteriology of water have been directed, for the most part, toward its sanitary aspects. The best single criterion by which the sanitary quality of a water may be judged is, clearly, the kind and numbers of bacteria that are present in it. If it were possible, as a routine procedure, invariably to detect the presence of the appropriate disease-producing bacteria, it would be unnecessary, from the sanitary point of view, to take the non-pathogenic forms into consideration. This is, however, not the case; a judgment of the sanitary quality of a water cannot be made on the basis of failure to find a microorganism such as the typhoid bacillus in that water. This bacterium, for example, is usually so outnumbered by similar forms such as the colon bacillus that its isolation and identification require enrichment cultures in selective media and other time-consuming procedures and even then are not always successful, and a negative finding is of doubtful value.

It is legitimate to assume, however, that when a water is polluted with human fecal material, it is probable that it contains bacteria which cause enteric infection. This probability becomes a certainty when the fecal material is pooled, as in the sewage of a community, because of the ubiquitous presence of healthy carriers of typhoid bacilli and similar microorganisms. Since it is im-

[1] Gorman and Wolman: Jour. Amer. Water Works Assn., 1939, 31:225.

[2] Prescott, Winslow and McCrady: *Water Bacteriology.* 6th ed. John Wiley and Sons, New York. 1946.

practical to isolate these pathogenic bacteria themselves as a routine measure, some indicator of fecal pollution will serve equally well as a criterion of the sanitary quality of a water. The question, which was stated many years ago,[3] becomes this: Are there types of bacteria which are never present in natural waters free of pathogenic bacteria but which may always be found in water polluted with human fecal material and therefore probably with pathogenic bacteria? If so such types of bacteria can be used as indicators of pollution. The answer to this question involves a consideration of the bacterial flora of natural waters, including both bacteria which are native inhabitants of water and those microorganisms whose presence is a consequence of contamination from external sources.

Bacteria Native to Natural Waters. The bacteria whose native habitat is water are not well known,[4] in part because many of them are difficult to grow on laboratory media. There is no question, however, of the existence of a bacterial flora normal to and characteristic of natural waters. The types of bacteria which make up this natural population may be considered briefly:

(1) *higher bacteria,* frequently the sheathed forms assigned to the *Chlamydobacteriales* and including sulfur, iron and other forms;

(2) the *Caulobacteria,* a relatively recently described group of "stem" bacteria which occur in lakes and other bodies of water attached to some inanimate object:

(3) the *spiral forms,* which are frequently found in great numbers in water, some of which may be very large, 20 to 30 μ in length, as compared with the parasitic spirilla:

(4) a variety of *bacilli,* including

    (a) pigmented forms such as *Bacterium prodigiosum, Bacterium violaceum, Bacterium aurescens,* and others,

    (b) various non-pigmented forms such as

        1. the fluorescent bacteria—*Pseudomonas fluorescens,*

        2. certain of the sulfur bacteria,

        3. thermophiles,

        4. aerobic, spore-forming bacilli of uncertain taxonomic position;

(5) *coccus forms,* both

    (a) pigmented, generally yellow—very often *Sarcina lutea,* and

    (b) non-pigmented—*Micrococcus aquatilis, Micrococcus candicans,* and others;

(6) *nitrogen-fixing bacteria—Azotobacter aquatile* in particular.

(7) *nitrifying bacteria—*both Nitrosomonas and Nitrobacter.

These water bacteria are found in fresh water in swamps, streams and lakes. The bacterial populations of salt waters have not been studied until recent years, but it appears[5] that the sea contains similar bacteria, including the nitrogen-fixing forms, the fluorescent bacteria, various pigmented forms and

[3] *Cf.* Jordan in Thirtieth and Thirty-first Repts. Mass. State Bd. of Health, 1898, 1899.

[4] *Cf.* the review by Baier: *Studien zur Hydrobakteriologie stehenden Binnengewässer.* Arch. f. Hydrobiol., 1935, 29:183, also the studies of Taylor: Jour. Hyg., 1940, 40:616; *ibid.,* 1941, 41:17; *ibid.,* 1942, 24:284.

[5] ZoBell and Feltham: Bull. Scripps Inst. Oceanog., Tech. Series, 1934, 3:275; Waksman, Hotchkiss, Carey and Hardman: Jour. Bact., 1938, 35:477; ZoBell: *Marine Microbiology.* Chronica Botanica, Waltham, Mass. 1946.

the like. The water bacteria bear the same relation to the constant and recurring transformations of organic matter that take place in natural waters as they do to the same processes in the soil and are, therefore, an integral part of aquatic life.[6]

Some, but by no means all, of the water bacteria can be cultivated on laboratory media. In any case no one medium suffices; the nitrifying bacteria, for example, cannot be grown in the presence of organic matter. Since the bacteria found on plates are of necessity only those that will grow upon the medium used, it will be clear that the value of plate counts as estimates of the numbers of these microorganisms is questionable. Enrichment cultures are sometimes useful, and it may be noted parenthetically that the simple holding of a water sample for a period of hours will frequently result in a marked increase in numbers. Considerable information has been obtained in recent years by a method of "slide culture" in which a clean glass slide is suspended in the water for several days, removed, stained and studied microscopically.

Bacterial Contamination of Natural Waters. In addition to the native water bacteria, a given water may, and usually does, contain a variety of bacteria as contamination from external sources. These sources are two—the air and soil, and human excreta.

Bacteria from the Air and Soil. The number of bacteria in the air bears, as might be supposed, a close relation to the quantity of larger suspended particles or "dust." There are fewer bacteria in the air of the country than of the city, fewer in mountain air than in the air of the lowlands, and the air in mid-ocean is nearly bacteria-free. Under appropriate circumstances bacteria suspended in the air are those expelled from the upper respiratory tract of human beings and are of no small significance in the transmission of "droplet infection," but in so far as air in general is concerned this is an insignificant and relatively rare occurrence. Pathogenic microorganisms, such as the tubercle bacillus and the pyogenic cocci, have been found in the air of hospitals and sickrooms, but as a rule pathogenic bacteria in dry dust are exceedingly rare. The kinds of microorganisms in the air vary somewhat in different localities, but certain forms are generally present. Molds and yeasts are quite common and in some instances outnumber the bacteria. These include various species of molds such as *Penicillium glaucum* (the blue-green mold) and various yeasts, many of them the pigmented (red) torulae. *Bacillus subtilis* and related forms, together with the various micrococci, often pigmented, are almost universally present. These organisms may be isolated from the air by various filtering devices and most efficiently by the air centrifuge of Wells. They find their way into natural waters through settling out or are swept down by rain.

Many of the bacteria present in the air, particularly the aerobic spore-formers, are essentially soil forms blown up in dust and able to survive drying. The soil itself contains tremendous numbers of bacteria; a gram of average field soil probably contains 100,000,000 to 50,000,000,000 living bacteria, most of which are found in the upper six inches of soil, few being found in undisturbed soil below a depth of four to five feet. The great majority of these

[6] *Cf.* Henrici in *Problems of Lake Biology*. Amer. Assn. Advancement of Science, Publication No. 10. 1939.

microorganisms are native to the soil and include the nitrogen-fixing and nitrifying bacteria, bacteria of the amylobacter group and a variety of forms whose biochemical activities are an integral part of the mechanism of the decomposition of organic matter in the soil.[7] A small part of the native bacterial flora of the soil consists of potential pathogens such as *Clostridium tetani*, *Clostridium edematis* (the bacillus of malignant edema), *Clostridium botulinum* and others.

Bacteria other than those which make up the normal flora of the soil may be present as contamination. For practical purposes the pathogens that may be present may be regarded as coming from one of two sources, the flesh of animals and of persons who have died of infectious disease, and the excreta of human beings. In the first instance only one organism, the anthrax bacillus, is of significance, for other bacteria causing diseases of animals and man, such as the causative agents of tularemia, plague, spotted fever and other diseases of animals, together with the bacteria of human disease such as the diphtheria bacillus, streptococci, etc., do not survive long in the soil (p. 127).

In the case of the anthrax bacillus, however, the spores are able to survive for long periods of time, perhaps years, and are brought to the surface from buried bodies by earthworms. Pastures may, then, remain infective for cattle for long periods of time.

The pathogenic bacteria contained in human excreta are, of course, those microorganisms which leave the body via the intestinal tract, *i.e.*, those causing the enteric diseases. These bacteria do not multiply in the soil but their vitality may be considerably prolonged, possibly for two or three months.

It will be clear that bacteria present in the air and in the soil have relatively ready access to bodies of water and contamination may take place either more or less continuously or at irregular intervals under certain unusual conditions, as during and immediately after heavy rains. The contribution of air and soil, the latter in particular, to the bacterial flora of water is, then, considerable. Since the bacteria native to water are not pathogenic, what of this contamination with respect to the sanitary quality of a water? Some of the potenial pathogens, *e.g.*, the bacilli of tetanus and malignant edema, are not infective when taken by mouth, and others, even though they survive for sufficient periods, rarely produce infection by this route. The normal portal of entry of the enteric bacteria is, however, the gastro-intestinal tract, and it is these microorganisms which constitute the significant contribution of contamination through the agency of soil.

The intestinal bacteria may be washed directly into lakes and rivers or other bodies of water by heavy rains; hence the presence of excreta on water sheds and consequent contamination of impounded waters is of considerable practical importance. In many instances, particularly in the case of shallow wells and the like, typhoid and other bacilli enter the water supply from privies, latrines and similar devices via ground water. The distance over which such contamination can travel is a function of the rate of death of the bacteria and the rate of ground water flow. The latter is obviously dependent upon many factors such as the amount of rainfall, the local geological formations and the

---

[7] Waksman: *Principles of Soil Microbiology*. Williams & Wilkins Company, Baltimore. 1st ed., 1927; 2nd ed., 1932.

10

like, and each instance of contamination or possible contamination must be considered individually. Fecal bacteria have been found to penetrate from 100 to 200 feet in ground water.[8] In general fine soils and sand tend to impede their progress to a greater extent than coarse sand and gravel. The rock formations may be of considerable significance; sandstone, for example, filters out bacteria, while limestone tends to erode with the formation of direct communicating channels, and wells drilled into such formations may contain typhoid bacilli which entered the water many miles away and are, therefore, always to be regarded as dangerous.

Contamination by Human Excreta. Contamination of water by human excreta may take place, not only indirectly through the agency of soil as noted above, but also directly. Such direct contamination is, for the most part, a consequence of human population densities and urban organization, and takes the form of dumping of sewage of one community into a body of water which serves as a water supply to another. Whether directly or indirectly contaminated, such waters contain not only the native bacterial flora supplemented by microorganisms from the soil, but also the bacterial flora of the human intestine. The contribution of the last consists primarily of *Bacterium coli* in very large numbers, together with *Clostridium welchii, Streptococcus fecalis* and the various intestinal pathogens.

Factors Influencing the Kinds and Numbers of Bacteria. The numbers of bacteria that may be found in a given water are dependent primarily upon the type of water, whether it is a *surface water,* such as that found in streams, lakes and shallow wells, or a *deep water* from deep driven wells. In the first instance opportunities for contamination are great and, as might be expected, many bacteria are present. The water from deep wells, on the other hand, has undergone an effective filtration in order to reach the deeper strata in which it is obviously not subject to any extensive contamination; hence relatively few bacteria are found.

A variety of environmental factors influences the bacterial content of water; chief among these are the amount of organic matter present and the temperature. In general, the more nutriment there is present in the form of organic matter, the greater the number of bacteria. Low temperatures are not conducive to rapid growth and tend to keep the numbers of bacteria down, a factor that favors the survival of pathogens such as the typhoid bacillus which are unable to multiply in any case. Higher temperatures result in an increase in bacterial numbers in the presence of sufficient organic matter, but if the supply of nutriment is not great, after a preliminary increase during which the food supply is exhausted, the numbers fall below the initial level. Other environmental factors are not infrequently influential in determining the types of bacteria present in a water; thermophiles will, of course, predominate in hot springs and sulfur bacteria in sulfur springs; and the acidity of many natural waters results in a limited flora of acid-resistant bacteria.

Bacteria in Ice.[9] Although it is difficult if not impossible to sterilize a substance by exposure to low temperatures, many of the bacteria present are killed, only the resistant cells surviving. The great majority of bacteria in water are

---

[8] See, for example, the studies of Caldwell: Jour. Inf. Dis., 1938, 62:225, 272.
[9] See Jenson: Food Research, 1943, 8:265.

killed by freezing. Hence ice always contains but a fraction of the number in the water from which it was formed. Over 90 per cent both of the ordinary water bacteria and of typhoid bacilli die within a few hours, and a progressive decline in numbers then takes place, less than 1 per cent of typhoid bacilli surviving at the end of a week of freezing. Ice stored for six months is practically sterile. Outbreaks of typhoid fever have rarely been traced to the use of ice, although in a few instances the evidence of ice transmission seems quite conclusive. Danger of typhoid infection from the use of ice in drinking water is, in the absence of direct contamination of the ice during handling, always less than from the use of water from the same source as the ice.

The Bacteriological Analysis of Water.[10] It will be apparent from the above discussion that the bacteria whose presence in water is a consequence of fecal pollution are not present in uncontaminated water and are sufficiently different from the native water bacteria that they may be readily distinguished. Of these bacteria *Bacterium coli* is present in greatest numbers, while *Streptococcus fecalis* and *Clostridium welchii*, although constantly present, are usually not so numerous.* It would appear, therefore, that any of these microorganisms could be used as an indicator of pollution. Of these *Bact. coli* is the most satisfactory, although both *Str. fecalis* and *Cl. welchii* have been used in Europe. The streptococci are, however, sometimes difficult to differentiate and die out more rapidly than coliform bacteria. *Cl. welchii* has the disadvantage that its spores remain viable over long periods of time in contrast to *Bact. coli* which, although more hardy than the typhoid bacillus, dies out in time; hence *Cl. welchii* does not allow the differentiation of recent and old pollution. The quantitative relations between the coliform bacteria and the enteric pathogens are discussed at some length by Kehr and Butterfield.[11]

The bacteriological examination of water for the presence of *Bact. coli* rests upon the fact that this microorganism ferments lactose. The standard procedure for the examination has been prepared jointly by the American Public Health Association and the American Water Works Association, and is revised at frequent intervals.[12] Briefly, it consists of three parts, (1) the presumptive test, (2) the confirmed test and (3) the completed test. In the first, lactose broth is inoculated with decimal dilutions of the water sample, commonly 10 ml., 1 ml. and 0.1 ml. (expressed as dilutions these are, respectively, 0.1, 1 and 10). The volume of the smallest inoculum producing fermentation provides a crude approximation of the numbers of *Bact. coli* present in the water. Of the selective enrichment media, containing ox bile and/or brilliant green or ricinoleate or lauryl sulfate, only lauryl sulfate tryptose broth has been officially accepted for the presumptive test without confirmation, and then not for filtered or treated waters. A more accurate estimate may be obtained by inoculating five tubes with each dilution and calculating the most probable number of *Bact. coli* on the basis of the number of tubes in which

[10] For British practice, which differs slightly from American, see Ministry of Health Rept. No. 71, *The Bacteriological Examination of Water Supplies*. His Majesty's Stationery Office, London. 1939.

[11] Kehr and Butterfield: Pub. Health Rep., 1943, 58:589.

[12] This procedure may be found in detail in *Standard Methods of Water Analysis*, American Public Health Association, 9th ed., 1946, and in condensed form in most of the standard laboratory manuals.

fermentation occurs. Tables for the calculation of the most probable numbers are given by Prescott, Winslow and McCrady.[2] The *confirmed test* consists of the inoculation of a specified selective medium such as Endo or eosin-methylene blue (EMB) plates, brilliant green lactose bile broth, crystal violet lactose broth, fuchsin lactose broth or formate ricinoleate broth. The appearance of typical *coli* colonies on the plates or fermentation in the selective lactose broth constitutes a positive confirmed test. In the *completed test* one or more typical colonies are picked from an Endo or EMB plate inoculated either from the original lactose broth culture or from the secondary selective medium showing fermentation and are transferred to an agar slant and a lactose fermentation tube. After incubation the slant culture is smeared and stained and examined for the gram-negative non-spore-forming rods of *Bact. coli*. If the culture is found to be morphologically *Bact. coli* and the lactose is fermented, the completed test is positive.

The Coliform Bacteria. Although *Bact. coli* is readily distinguished from the native water bacteria, the closely related *Bacterium aerogenes* is found in grains and elsewhere in nature though not, it may be noted, in virgin soils. The two bacterial species may be differentiated by a number of tests (p. 422), but these are not a part of the standard method of water examination; hence the gram-negative, lactose-fermenting bacteria whose presence is determined are more properly termed *coliform bacteria* rather than *Bact. coli*.

The Sanitary Significance of Coliform Bacteria. In recent years there has been a tendency in sanitary water analysis to emphasize the distinction between *Bact. coli* and *Bact. aerogenes,* the presence of the latter being considered by many to have little or no sanitary significance. There is no doubt that *Bact. coli* is more predominantly of "fecal origin" while *Bact. aerogenes* is found in greater relative abundance in soil than in sewage. For this reason it is maintained that a predominance of *Bact. aerogenes* over *Bact. coli* in a water supply is more indicative of soil contamination (the *coli* presumably of animal origin) or past pollution than of recent pollution. In general, however, while the proportion of *Bact. coli* to *Bact. aerogenes* is frequently correlated with the sanitary survey, there are too many exceptions to warrant attaching great significance to it. The ecology and significance of the different types of coliform bacteria found in water are reviewed and considered in detail by Taylor.[13]

It should be remembered, however, that coliform bacteria of all kinds are practically absent from virgin soils and from pure spring and surface waters, and that while *Bact. aerogenes* and intermediate forms are not present in feces in as great numbers as *Bact. coli,* their presence may nevertheless be demonstrated by appropriate methods.[14] Even if it is true that *Bact. aerogenes* is somewhat more resistant than *Bact. coli,* and hence may survive in soil or water long after the latter has disappeared, the fact that its presence is not reliable evidence of *recent* pollution may not be as decisive as sometimes assumed. Experience in water examination has shown that it is not safe to disregard the warning of potential danger conveyed by evidence of soil washings and "past pollutions." It is unwise, therefore, in routine water analysis, to place

[13] Taylor: Jour. Hyg., 1942, 42:23.
[14] Gray: Jour. Hyg., 1932, 32:132; Bardsley: Jour. Hyg., 1934, 34:38.

too much stress upon the differentiation between *Bact. aerogenes* and *Bact. coli*, at all events until the practical value of such differentiation can be clearly demonstrated.

Plate Counts. It is usually desirable to have an approximate measure of the total number of bacteria in drinking water, not because the sanitary quality of a water can be judged on this basis alone, but because such information frequently has ancillary value. The counts obtained by quantitative dilution and plating are, it must be remembered, those of the microorganisms that will grow on the medium used, other bacteria being quite inapparent by this method.

Two series of plates are poured, one in which the medium is nutrient gelatin and the other nutrient agar, or both may be nutrient agar. The gelatin plates, or one set of agar plates, are incubated at 20° C. and the agar plates at 37° C.[12] In general the native water and soil bacteria grow best at 20° C.; in some cases they do not grow at all at 37° C.; and bacteria of animal origin grow most rapidly at body temperature. The relative numbers of microorganisms growing at the two temperatures are, then, at times suggestive of the origin of the bacteria found.

Chemical Analysis. The analysis for appropriate chemical compounds frequently is of value as an adjunct to bacteriological analysis in the determination of the sanitary quality of a water.[12] Pollution by sewage, for example, adds complex compounds, protein, carbohydrate and fat, to the water, and the amount and state of the decomposition products of these substances may serve as an index of the degree and time of pollution. Ammonia, nitrites, nitrates, chlorides and albuminoid nitrogen are usually determined. Of these, chloride, and to a certain extent nitrate, are the most useful. It may be noted that the chemical analysis of water is frequently made in connection with hardness, turbidity, taste, smell and similar features, which, while often of considerable industrial or esthetic significance, are of no sanitary importance.

The Assay of the Sanitary Quality of Water.[15] The means by which the sanitary quality of a water is judged may be summarized briefly:
- (1) The bacteriological analysis, including both
    - (a) the presence or absence of coliform bacteria and
    - (b) the number and type of bacteria present;
- (2) the type of water, whether surface or deep;
- (3) the local conditions; and
- (4) chemical analysis.

Of these the presence and numbers of coliform bacteria are the most important, and it must be remembered that the relative abundance, rather than the presence, of these microorganisms is the essential feature of the test. The discovery of a single colon bacillus in 50 ml. of water, or even occasionally in 5 ml., affords no reasonable ground for suspicion of the water. The possibility of sporadic contamination with colon bacilli derived not from man but from domestic animals or birds must be kept in mind. Manured fields and pastures, filled with grazing cattle or sheep, are likely sources of colon bacilli and may give rise to mistaken inferences if the environmental examination of a water supply is

[15] Streeter: *Laboratory Control of Water Supplies.* Public Health Reports, Suppl. No. 201, 1948.

neglected. Knowledge of such local conditions as well as the type of water is, then, essential to the interpretation of the bacteriological findings. Chemical analysis may be of considerable help in some instances, but in general finds its greatest utility in the study and control of gross pollution in which the decomposition of organic matter and presence of industrial wastes are a nuisance, rather than the assay of the purely sanitary quality of a water.

Drinking Waters. The question of the significance of the bacteriological findings brings up the matter of standards to which a water suitable for drinking should conform. Now it will be clear that, although considerable numbers of colon bacilli in a water are always suggestive of fecal contamination by man or animals, a standard is necessarily a minimum which is inherently difficult to define under the circumstances. In this country the United States Public Health Service has prepared recommended standards[16] which are intended to represent a minimum. In the recommended procedure the frequency of sampling is dependent upon local conditions, the minimum varying from one per month for a population of 2500, to 500 per month for a population of 5 million. The sample is 5 10 ml. portions, or 5 100 ml. portions. The results of bacteriological examination by Standard Methods procedures should be as follows:

(1) Of all 10 ml. portions examined per month, not more than 10 per cent shall show the presence of coliform bacteria.
(2) Occasionally more than 3 of the 5 samples show coliforms; this must not occur in more than 5 per cent of samples when 20 or more samples are taken per month, or in more than 1 sample if less than 20 samples are taken per month.
(3) Should such a result (as in 2) be obtained from a single standard sample, daily testing must be carried out until at least 2 consecutive satisfactory samples have been found. Such daily samples are to be regarded as "special samples" and not included in the monthly totals.
(4) With regard to the 100 ml. samples:
    (a) Not more than 60 per cent shall show coliforms.
    (b) Occasionally all 5 portions constituting a single sample will show coliforms; this must not occur in more than 20 per cent of samples when 5 or more samples are examined per month, or in more than 1 if less than 5 samples are taken per month. If so, daily "special samples" must be taken as above.

The water shall be satisfactory as to taste, odor and color and shall contain less than the following minimum quantities of chemical impurities: lead, 0.1 ppm; fluorine, 1.5 ppm; arsenic and selenium, 0.05 ppm; copper, 0.3 ppm; iron and manganese, 0.3 ppm; magnesium, 125 ppm; zinc, 15 ppm; chloride and sulfate, 250 ppm; total solids, 500 ppm; phenol, 0.001 ppm.

The British Ministry of Health suggests standards[17] based on the presumptive coliform count as determined by acid and gas formation in MacConkey broth on the piped supply entering the distribution system. Waters are divided into classes on the following basis:

Class I—Water of class I and regarded as highly satisfactory contains less than 1 coliform per 100 ml.

[16] United States Public Health Service: *Manual of Recommended Water-Sanitation Practice.* Pub. Health Bull. No. 296, 1946. These standards are also summarized in Pub. Health Rep., 1946, 61:371; and in Jour. Amer. Water Works Assn., 1946, 38:361.
[17] *The Bacteriological Examination of Water Supplies.* Ministry of Health Series No. 71. His Majesty's Stationery Office, London. 1939.

Class II—Water regarded as satisfactory contains 1 to 2 coliforms per 100 ml.
Class III—Water regarded as suspicious contains 3 to 10 coliforms per 100 ml.
Class IV—Water regarded as unsatisfactory contains more than 10 coliforms per 100 ml.
A water of satisfactory sanitary quality should show at least 50 per cent of samples in class I, not more than 80 per cent below class II, and none below class III.

While a presumptive test alone is specified, the occurrence of positive presumptive tests has been found to be very high in Britain. Waters of classes I and II conform closely to the American standards.

Swimming Pools and Bathing Places. The sanitary control of water in swimming pools and bathing beaches is similarly based on bacteriological examination. The American Public Health Association has recommended that not more than 15 per cent of samples of swimming pool water contain more than 200 bacteria per ml. or give positive confirmed tests for coliforms in any of 5 10 ml. samples when the pool is in use. The standard is as high as that for drinking water, but pools are usually filled with water of drinking quality and pollution is not only derived from bathers but is fresh and may be highly infective. The presence of acid-forming streptococci is also of considerable utility as a measure of oral and skin contamination, and usually corresponds closely to the total count. Such waters commonly contain residual chlorine which must be neutralized with thiosulfate when samples are collected for bacteriological examination. In natural outdoor bathing places the test for coliforms is the most important. The standards are necessarily much more lenient than those for indoor pools, and those adopted locally vary from an allowable 100 coliforms per 100 ml. in California and Indiana to 3000 coliforms per 100 ml. allowed by the New York City Health Department.

The Purification of Water Supplies. When, by bacteriological examination or otherwise, a water is known to be unsafe for consumption, the question arises as to ways and means of artificial purification. There are a number of useful methods of purifying water, differing according to the amount and character of the water to be treated, which may be summarized as follows:

(1) mechanical methods
    (a) storage
    (b) filtration
        1. slow sand filtration
        2. coagulation and rapid sand filtration
(2) chemical methods
    (a) large scale—hypochlorites and liquid chlorine
    (b) small scale—hypochlorite, ultraviolet light, ozone, etc.

Of these, storage is not generally regarded as a method of water purification, though the numbers of bacteria are usually greatly reduced in impounded waters because of the exhaustion of the food supply and the consequent death of bacteria and settling, not so much of bacteria alone as of suspended matter which carries down bacteria with it. The partial removal of suspended matter is frequently desirable, particularly with turbid waters, and may be carried out by allowing the water to remain in a settling basin for a time.

Slow sand filtration is one of the earliest and most effective methods of water purification and is in use in many European and some of the older American

cities. These sand filters are constructed so that the water passes through 1 to 5 feet of sand supported upon graded layers of gravel (see Fig. 33). The rate of filtration must be accurately regulated and the efficiency of operation controlled by frequent bacterial tests of the effluent. Such filters are highly effective. Bacteria are removed, not to any great extent by mechanical straining out, but through a biological mechanism in which the activity of protozoa is an important feature. The passage of water through these filters is necessarily a relatively slow process and, in consequence, relatively large areas are required, which, for financial or other reasons, are no longer available in large American cities. Few of these filters have been constructed in recent years, and the use of rapid sand filters is becoming common.

The rapid sand filters, which may be used with turbid waters that would clog a slow sand filter, are frequently employed in conjunction with "coagulation," the addition of such substances as aluminum or ferric sulfate, which form flocculent precipitates (the hydroxides). The precipitate carries down most of the suspended matter and, of course, many bacteria, and is readily filtered out,
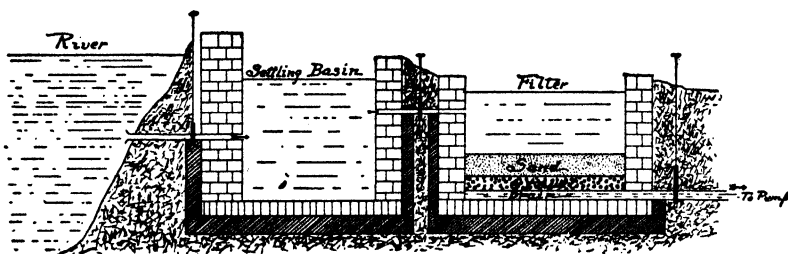
Fig. 33. Cross-section of filter plant (after Hazen).

yielding a clear effluent containing relatively few bacteria. Laboratory experiments on centrifugation of dilute suspensions of *Bact. coli* in the presence of kaolin or infusorial earth have indicated that 70 per cent of the viable cells are found in the sediment.[18] Such filters must be cleaned from time to time, a process that is seldom required with slow sand filters. They make possible, however, the treatment of a large quantity of water on a relatively small filtering area.

Destruction of pathogenic bacteria in water supplies is most often brought about by treatment with germicidal chemicals. Of these, hypochlorite (calcium hypochlorite or bleaching powder) was at one time used widely in the treatment of municipal water supplies, and it still has its uses, although it has been largely superseded in large scale treatment by liquid chlorine, which is supplied in cylinders under pressure. Chlorine is added directly to the water through an automatic feeding device in accurately measured amounts which are determined by the character of the water. In general, the greater the amount of extraneous organic matter present, the greater must be the amount of chlorine added. The amount of chlorine taken up is termed the chlorine demand, and the point at which the residual chlorine or available chlorine becomes proportional to the added chlorine is called the "breakpoint" in the chlorine demand curve. The introduction of breakpoint chlorination usually

[18] Schulhoff and Huekelekian: Jour. Amer. Water Works Assn., 1936, 28:1963.

results in material improvement in the sanitary quality of a water.[19] When liquid chlorine is added to ordinary surface water, clear and not highly contaminated, in the proportion of about 0.5 to 1 part of "available chlorine" per million gallons, the ordinary intestinal bacteria are destroyed, including such pathogenic forms as the typhoid bacillus.[20] The tastes and odors in chlorinated waters may be due to an overdose of chlorine caused by inadequate control methods, or to the action of chlorine upon compounds present in the water, commonly as industrial wastes. Excess chlorine and tastes caused by chlorination can often be removed by dechlorination with SO_2 or treatment with $KMnO_4$ or activated carbon. The bactericidal activity of chlorine may be prolonged, particularly in waters containing considerable organic matter, by the simultaneous addition of liquid ammonia with the formation of chloramines.[21] The introduction of chlorination of municipal water supplies has, in practically every instance, resulted in marked reductions in the incidence of enteric infection, typhoid fever in particular, in this country. The addition of chlorine or other bactericidal agents is not a "cure-all" however. When a water is sufficiently heavily contaminated, bacteria embedded in particles of organic matter are not killed even in the presence of available chlorine. It is generally agreed that a content of more than 50 coliform bacteria per milliliter indicates pollution too great for successful chlorination.

In the last few years chlorine dioxide (ClO_2) has been applied to the treatment of water. It has the advantages that it destroys algal tastes as well as the chlorophenol taste of certain chlorinated waters, and oxidizes organic matter more rapidly than chlorine, thus allowing the maintenance of a chlorine residual in the distribution system. In water solution this substance is decomposed by light to chloric and perchloric acids and oxygen, and is more bactericidal for coliform bacteria than chlorine.[22]

It may be noted that frequently a combination of filtration and chlorination is desirable particularly with rapid sand filters. Not only does a preliminary filtration have esthetically desirable features in the case of turbid waters, but considerably less chlorine is required for treatment than would have been the case had not the greater part of the suspended material been removed.

The chemical treatment of water on a small scale does not always involve expense as a primary consideration and hence other methods are not infrequently used. The treatment of swimming pool water by ultraviolet irradiation adds no tastes or odor to the water and, although considerably more expensive than chlorination, can be used because the scale of operation is small. Ozone, which is strongly bactericidal, is also relatively expensive and is not commonly used in this country but is used to an appreciable extent in Europe.[23] Chlorina-

[19] *Cf.* Griffin and Chamberlin: Amer. Jour. Pub. Health, 1945, 35:199.

[20] See, for example, the studies of Levine, Heller and Bender: Jour. Amer. Water Works Assn., 1942, 34:1787.

[21] The chlorine-ammonia treatment is discussed in Jour. Amer. Water Works Assn., 1941, 33:2079.

[22] See Synana, McMahon and Vincent: Water Works and Sewerage, 1944, 91:423; *ibid.*, Jour. Amer. Water Works Assn., 1945, 37:869; *ibid.*, Amer. Jour Pub. Health, 1946, 36:1035.

[23] See the review on ozone treatment by Hann: Jour. Amer. Water Works Assn. 1943, 35:585.

tion is, of course, always available. It is carried out by the addition of hypo-chlorite when the installation of liquid chlorine apparatus is not desirable, as, for example, in the treatment of a contaminated cistern.

At times the treatment of water becomes an individual matter, as in the case of an army in the field or when a public supply is known to be impure. In the first instance the water may be treated by the addition of hypochlorite in the form of bleaching powder or a solution of sodium hypochlorite, or by the addition of a small amount of iodine, one part of Lugol's iodine solution to 1000 parts of water. In the home, filters, such as the Berkefeld or Chamber-land bougies, may be used, but filtration is slow and care in operation and frequent cleaning are necessary. The simplest and best method of water treat-ment for the family or individual, however, is simple boiling. Boiling for five minutes is quite sufficient to destroy with certainty the typhoid bacillus and allied forms as well as the cholera vibrio. When water-borne disease is prev-alent, or when a water supply is notoriously impure or exposed to chance of infection, boiling is the only wholly safe procedure.

SEWAGE

Sewage is best regarded as the used water supply of a community and as such is a dilute solution of fecal matter and other wastes. From the hygienic point of view it is an important vehicle in the transmission of enteric infec-tion; hence the manner of its disposal is of considerable significance. The mechanisms of sewage disposal have as their objects first, the ridding of a community of an ever-present volume of waste and second, disposal in such a manner that it is not dangerous to other communities.

The complex organic compounds present in sewage undergo the same proc-esses of decomposition that are involved in the breakdown of dead organic matter in nature and are a part of the so-called cycles of elements such as nitrogen, phosphorus and the like. Any type of sewage treatment, then, is nothing more than a mechanism for bringing about or accelerating these trans-formations. The organic compounds are first broken down to amino acids, monosaccharides and the like, which are eventually oxidized completely to carbon dioxide and water in the case of carbon and hydrogen, and to nitrite and nitrate in the case of nitrogen. Bacteria are the active agents in this decomposition and oxidation, the mechanisms of which have been discussed earlier (Chapter 4). Although essentially simple in principle, sewage treat-ment is in practice a complex problem which cannot be considered at length here.[24]

In general, however, sewage disposal falls into one of three categories: (a) dilution, (b) partial treatment and (c) complete treatment. In the first in-stance the sewage is simply dumped into some body of water where it will not annoy its originators. Here the breakdown and oxidation of the constituents of the sewage occur in nature, and, if sufficient time elapses, no trace remains beyond an increase in nitrate which, in turn, serves as food material for phyto-plankton. When this transformation takes place in a flowing stream, the phe-nomenon is known as the *self-purification of streams*. The essential element, however, is not the fact that the water is in movement but that sufficient time

[24] See Imhoff and Fair: *Sewage Treatment*. John Wiley & Sons, New York. 1940.

elapses for the breakdown and oxidations to proceed to completion. Soil polluted with sewage similarly "purifies itself."

With increasing population densities the disposal of sewage by dilution becomes unsatisfactory because a body of water is not infrequently a water supply to a neighboring community. Some form of treatment, then, becomes obligatory; in other words, the decomposition is made to take place in whole or in part in the various tanks and other devices making up a sewage treatment plant rather than allowing it to occur in natural bodies of water. In practice, treatment takes the form of preparatory processes followed by a period of anaerobic digestion, then a period of aerobic oxidation. Treatment may be complete even through nitrification, or the partially treated sewage may be disposed of by dilution.

When the disposal of fecal material is an individual or family problem the mechanics involved are somewhat different, but the processes of decomposition and oxidation are essentially the same whether a privy, cesspool or septic tank is used.

It will be noted that, although sewage treatment is basically a bacteriological process, the pathogenic bacteria are not involved; their presence is not taken into consideration, nor is there any effort to destroy them except in the rare instances in which the effluent from a complete treatment plant is chlorinated. In one sense, then, sewage treatment is not directed toward the control of water-borne disease. The fact that it is treated, however, is of considerable significance in this connection. When sewage is disposed of by dilution, the typhoid bacillus and related microorganisms do not multiply; rather, a decline in numbers sets in immediately, and these bacteria do not survive as long in water as in soil, probably not more than a few days or a week. It is probable that they tend to disappear equally rapidly during sewage treatment, although there is no assurance that pathogens are absent in the effluent from treatment plants. Clearly then, fresh pollution is more dangerous than past pollution, and, in a sense, treatment obviates the possibility of such fresh pollution. The economic and esthetic aspects of sewage treatment are, of course, another matter.

THE BACTERIOLOGY OF MILK AND FOOD

MILK[1]

As a vector of infectious disease, milk differs from water in that it is an excellent medium for the growth of many pathogenic bacteria, and from other foods in that it is the only food of animal origin that is consumed in large part in the raw state. Since large quantities of milk are consumed—it is estimated that about 16 per cent of the average dietary in the United States consists of milk and milk products—the importance of this substance in the transmission of disease is evident. The diseases transmitted by milk are: first, diseases of cattle transmissible to man and including bovine tuberculosis, undulant fever, foot-and-mouth disease and streptococcal infections from infected udders; and, second, diseases of man in which the milk serves as the link between man and man, such as typhoid fever, septic sore throat, scarlet fever, diphtheria and, rarely, certain other diseases, such as poliomyelitis.

Sources of Bacteria in Milk. Unlike water, milk has no native bacterial flora, and it is probable that milk as secreted into the udder of a healthy cow is sterile. The milk in the udder is, however, rarely if ever bacteriologically sterile, for the microorganisms invade the udder via the milk ducts of the teats and the first portion of the milk drawn (fore-milk) always contains more bacteria than the last (strippings). There is, furthermore, no bacterial flora that is characteristic of milk; the presence of microorganisms is always a consequence of contamination and the types of bacteria found are determined by the source of contamination. From this point of view the bacteria of milk fall into two groups: first, those which are present in the tissues of an infected cow and find their way into the udder; and, second, those which enter the milk, usually after it is drawn, from sources external to the animal.

Bacteria from Infected Cattle. Of these microorganisms perhaps the most important is the *tubercle bacillus*. These bacteria get into the milk directly as a consequence of tuberculosis of the udder, which occurs in 1 to 2 per cent of infected cows, and indirectly by contamination with cow manure when the infectious sputum is swallowed and discharged with the feces. In either case the milk is infective for man, the bovine variety of the tubercle bacillus giving rise, as a rule, to bone and joint rather than pulmonary tuberculosis (p. 631). Milk may, of course, be infected with human tubercle bacilli from infected persons. A high proportion of the former type of infection occurs when the transmission of this disease is allowed to occur. The disease may be controlled at its source, *i.e.*, by the elimination of infected cattle from dairy herds, a practice which is practically universal in the United States.

[1] Hammer: *Dairy Bacteriology.* 3rd ed. John Wiley and Sons, New York. 1948.

A bovine variety of *Brucella melitensis*, the causative agent of undulant fever (Chapter 23), infects cattle, producing the disease contagious abortion (the bacterium is sometimes designated *Brucella abortus*). This microorganism is excreted in the milk and infects man, producing a mild type of undulant fever. The caprine variety of this bacterium which is present in the milk of infected goats produces a much more severe disease in man. Undulant fever, like tuberculosis, may be controlled by control of the disease in the animal reservoir of infection.

The virus of foot-and-mouth disease, a virus disease of cattle, is excreted in the milk and is thus transmitted to man. The disease in man is mild, however, and not of great public health importance. More important are the streptococcus infections of the udder, designated garget or mastitis. The *Hotis test* is commonly used for the detection of mastitis; it consists of incubating fresh milk in the presence of 0.025 per cent bromcresol purple for twenty-four hours at 37° C. A positive reaction, the formation of yellow flakes on the side of the test tube, is dependent upon the presence of the streptococci and of agglutinins in the milk; the growing bacteria are clumped by the antibody and the acid reaction results from the fermentation of lactose. The indeterminacy of streptococcus species, coupled with the fact that the same type of streptococcus may produce more than one disease (p. 367), makes it difficult to evaluate the significance of streptococcus mastitis to milk-borne disease. Milk-borne epidemics of septic sore throat, however, are not infrequently associated with acute udder inflammation in the dairy herd and the massive and continuous infection occurring in some of the outbreaks indicates that the udders of the cattle were infected. It has been suggested that the streptococci of scarlet fever may proliferate, with or without symptoms, in the udder, and epidemics of human streptococcal infection, septic sore throat and scarlet fever, may occur,[2] though it is probable that in most instances milk-borne scarlet fever is a consequence of direct human contamination. It may be noted that the udder may be infected occasionally with diphtheria bacilli, which produce small external ulcers, but this is an uncommon occurrence.

Bacteria from External Sources. When milk is collected under ordinary conditions, the udder bacteria form but an insignificant fraction of the total number of microorganisms in the milk. The skin of the cow, the hands of the milker, the vessels used for collection, and the dust of the cow barn all contribute their quota to the number of bacteria found immediately after milking. If milk is obtained with aseptic precautions, it contains only a few hundred (200 to 400) bacteria per milliliter; collected with somewhat less care, it may contain a few thousand (2000 to 6000); with careless manipulation, even freshly drawn milk may be highly contaminated (30,000 to 100,000 per milliliter). If milk is kept at 0° C. (32° F.), it shows a decrease in the bacterial content during the first few hours, but at higher temperatures the rate of multiplication is high and, when richly seeded at the outset, enormous numbers of bacteria result.

The non-pathogenic bacteria that are present in milk are often differentiated on a physiological basis into the following groups:

(1) the acid-forming bacteria,

[2] Evans: Jour. Inf. Dis., 1946, 78:18.

(2) the alkali-forming bacteria,

(3) the proteolytic bacteria and

(4) the inert bacteria.

The first group includes the fermentative bacteria, and the most common type of fermentation is the lactic acid fermentation, the process by which milk usually sours under natural conditions. A variety of bacteria may be responsible, among them such familiar forms as *Staphylococcus aureus, Streptococcus pyogenes* and *Bacterium coli.* A few species, however, are commonly active in the natural souring of milk, and these may be divided into two groups. One of these is comprised of the capsulated gas-forming bacilli of the *Bacterium (lactis) aerogenes* type, which are closely related to *Bact. coli,* differing principally in their possession of capsules, lack of motility, and ability to produce gas from potato starch. The second type, a streptococcus, *Streptococcus lacticus* (or *lactis*), is abundant in naturally soured milk, particularly when the acidity is high.

Lactic acid milks, regarded by some as having therapeutic value in the treatment of certain intestinal disorders, have been prepared by the inoculation of *Lactobacillus* species such as *acidophilus* and *bulgaricus.* The lactobacilli, however, are commonly found in the fermentation of ensilage and are present in small numbers in the human mouth and intestinal tract and are not ordinarily responsible for the natural souring of milk.

Although lactic acid is commonly the predominating acid in the fermentation of milk, the formation of butyric acid is observed occasionally. This is generally a consequence of the presence of the anaerobic butyric acid bacteria, but may be brought about through the agency of certain aerobes closely allied to *Bacillus subtilis.*

The spontaneous alcoholic fermentation of milk is less usual under natural conditions than either the lactic or the butyric, and the preparation of certain alcoholic beverages is dependent upon the artificial production of this form of milk fermentation. Koumiss, a drink prepared by Tartars by the alcoholic fermentation of mare's milk, and kefir, an effervescent sour milk prepared by inhabitants of the Caucasus from the milk of cows, goats and sheep, are both prepared by the inoculation of fresh milk with old koumiss in the first instance and with "kefir granules" in the second. The bacteriology of the koumiss fermentation is not well known, but in the case of kefir both a bacterium and a yeast appear to be involved. Some species of yeast, it may be noted, are able to effect the alcoholic fermentation of milk in pure culture.

The alkali-forming bacteria are those organisms which do not ferment lactose but, presumably, act upon the nitrogenous substances present with the liberation of ammonia. When typhoid and paratyphoid bacilli, for example, are cultivated in litmus milk no effect is apparent beyond a slowly increasing alkalinity. Certain other bacteria, such as some of the aerobic spore-forming types, also produce lipase and decompose the fats present, converting the milk to a yellow, transparent fluid.

The proteolytic bacteria also produce an alkaline reaction and, in addition, hydrolysis of the milk proteins. Two enzymes, or groups of enzymes, are responsible; one, a rennin-like enzyme, precipitates the protein with the formation of a soft curd, and a second, casease, brings about hydrolysis of the protein

which, when complete, results in the conversion of the milk to a clear fluid, a process sometimes termed peptonization. Peptonization, however, does not follow precipitation when the microorganism does not possess casease. The bacteria producing these changes include spore-forming aerobes such as *Bacillus subtilis*, certain strains of staphylococci, *Proteus vulgaris* and others. Proteolysis may occur in heated milk in which the non-spore-forming lactic fermenters have been destroyed, leaving the more resistant spore-forming proteolytic forms.

The "Diseases" of Milk. A series of unusual or abnormal changes, sometimes called "diseases" of milk, are produced by certain bacteria which occasionally find their way into milk. "Blue milk" (*Bact. cyanogenes*), "red milk" (*Bact. prodigiosus*, *Bact. erythrogenes, et al.*) and "yellow milk" (*Bact. synxanthus*) are caused by the presence of various chromogenic bacteria. "Bitter milk," characterized by a bitterness that sometimes develops after a short interval, is likewise due to the products of certain microorganisms. Milk sometimes suffers from a ropy or slimy fermentation which, under most circumstances, is regarded as undesirable, although such a fermentation is intentionally produced in the manufacture of Edam cheese in Holland through the action of a particular species of streptococcus.

The *inert bacteria* are those which produce no visible change in milk. These include certain non-pigment-forming bacteria from water and other sources, and, in addition, most of the pathogenic bacteria that find their way into milk. Such dangerous contamination, then, is inapparent without bacteriological examination.

The Pathogenic Bacteria from External Sources. The multiplicity of sources of contamination results in the heterogeneous bacterial flora that may be found in milk. It is important from the hygienic view that, in addition to microorganisms of soil and water, the bacteria carried by man have relatively ready access to milk. The opportunity for contamination with the bacteria of human disease is, then, ever present, not only when the milk is drawn but throughout its handling until it reaches the ultimate consumer. Contamination may take place directly, as in the case of scarlet fever and other streptococci or diphtheria bacilli from the throat and typhoid bacilli from the hands of infected individuals, or it may be indirect, as when the water used to wash the milk cans is contaminated with typhoid bacilli. In any case, the microorganisms do not simply survive, as in water, but actively multiply and may be present in huge numbers in pooled milk of which but a single part was originally contaminated.

Clearly, then, the most important factors which govern the number of bacteria which may be present in milk are, first, the kind and degree of initial contamination and, second, the temperature at which the milk is kept. The production of hygienically satisfactory milk, therefore, involves cleanliness in the first instance, and immediate cooling and storage at a low temperature in the second. Practical experience has more than adequately proved the significance of these points.

The Bactericidal Property of Fresh Milk. As indicated above, the number of bacteria present in freshly drawn milk frequently shows an initial decrease. The extent of this diminution is, perhaps, exaggerated by plate

counts; the various antibodies are present in the milk, not only the bactericidal substances but the agglutinins as well, which, by clumping the bacteria, may bring about decreased plate counts. That freshly drawn milk is both bactericidal and bacteriostatic, albeit to a mild degree, is, however, definitely established. This activity is thermolabile, being destroyed in fifteen minutes at 75° C. and in two minutes at 80° to 90° C., and disappears a few hours after the milk is drawn.

MILK-BORNE EPIDEMICS OF DISEASE*

Disease	Outbreaks		Cases	
	Number	Per Cent of Total	Number	Per Cent of Total
Typhoid and paratyphoid fever..............	76	45.2	1,209	12.1
Septic sore throat and scarlet fever..........	57	34.0	6,812	68.2
"Gastro-enteritis".......	24	14.3	1,423	14.2
Bacillary dysentery......	5	3.0	411	4.1
Diphtheria.............	5	3.0	123	1.2
Poliomyelitis...........	1	0.6	11	0.1
Totals...............	168	100.0	9,989	100.0

* In New York State exclusive of New York City as reported by Dublin, Rogers, Perkins and Graves: Amer. Jour. Pub. Health, 1943, *33*:157.

The Determination of the Quality of Milk. By far the best index of the quality of milk is the number of bacteria it contains. The bacteria present are, in all cases, a result of contamination, and hence these micro-organisms are reliable indicators of cleanliness and care and are generally used for this purpose.

Plate Counts. The total bacterial count of a milk is, then, a reflection of its hygienic quality, and the plate count has been, and still is, widely used in the bacteriological grading of milk. Standardization of the media and of the technique is, of course, necessary for comparable results. A standard procedure has been developed under the auspices of the American Public Health Association, whose publication[3] may be consulted for the details.

Standards of milk quality in terms of plate counts are in general use but vary from one locality to another. In some instances more than one grade of milk is allowable; for example, a "Grade A" milk may contain not more than

[3] *Standard Methods for the Examination of Dairy Products.* American Public Health Association. 9th ed., 1948. For a discussion of current practical application of these methods see Black: Pub. Health Rep., 1943, *58*:1605, 1641, 1681.

30,000 bacteria per milliliter as delivered, and a "Grade B" milk not more than 100,000. There is a steady movement toward more strict requirements, and the maximum number of bacteria allowed is continually reduced. More than one grade of milk is, however, undesirable in that the distribution of inferior milk is allowed, and, when possible, a single standard is preferable. In Chicago, for instance, there is but one grade of milk, and it must not contain more than 10,000 bacteria per milliliter as delivered to the consumer.

Microscopic Counts.[4] Besides the ordinary macroscopic colony count obtained by plating, a microscopic method devised by Breed is of value for many purposes. In the Breed method milk is taken in a capillary pipette discharging 0.01 ml. and is dried over an area of one square centimeter on a glass slide. After washing out the fat with xylol and fixing with alcohol, the film is stained with methylene blue. The number of bacteria per square centimeter is estimated by counting a carefully measured area. The ratio used in comparing the microscopic count with the standard plate count is 4 : 1, although it is recognized as inaccurate and variable. This direct microscopic count does not involve incubation and has proved of special value in judging the quality of fresh milk as delivered to milk-receiving stations.

Methylene Blue Reduction. If a small amount of methylene blue is added to milk and the mixture incubated, the dye will, in time, be reduced to the colorless leuco base. Although freshly drawn milk has some small power to reduce this and other dyes, the reduction is, for all practical purposes, a consequence of the metabolic activities of contained bacteria. There is, then, a direct relation between the length of time required for reduction and the number of bacteria present, and the measurement of this reduction time provides a useful approximation of the quality of a milk. The method is best adapted to raw milk and has the great advantage that no special apparatus or training of the operator is required. Although milk cannot be graded with any degree of precision in this way, it is generally agreed that a milk decolorizing in less than two hours is poor in quality, while that which does not decolorize in eight hours is excellent. Resazurin has been used to some extent in the place of methylene blue.

Bacterium Coli Count. It may be assumed that since *Bact. coli* may be used as an index of the sanitary quality of water, it could occupy a similar position with regard to milk. It will be recalled, however, that water contamination of sanitary significance is fecal, whereas this is not the case with milk; *Bact. coli*, for example, would not be associated with the presence of the diphtheria bacillus in milk. Furthermore, since the great majority of market milks contain cow feces, the estimates varying from 60 to 100 per cent, *Bact. coli* in milk is probably most often of bovine origin. Although the *coli* count has been used widely in the past, the differentiation of these microorganisms is not worth while except under special circumstances that warrant it.

The Isolation of Pathogenic Bacteria. In contrast to water, the isolation of pathogenic bacteria from milk is not only a practical but often a desirable procedure. The presence of tubercle bacilli, for example, can be conclusively shown only through the isolation of these microorganisms, in this instance usually by guinea-pig inoculation. Other pathogens, such as hemolytic strepto-

[4] For a general discussion see Brew and Breed: Amer. Jour. Pub. Health, 1945, 35:683.

cocci, Brucella, etc., may be isolated by culture. The isolation of these bacteria is not, of course, a routine procedure but is frequently carried out when an outbreak of disease is suspected of being milk-borne. The details of the methods employed may be found elsewhere.[3]

Cell Count and Sediment Test. The number of leucocytes present in a sample of milk and the amount of contained dirt that can be strained out through a standard cotton disc are frequently of value in the assay of milk quality. Excessive numbers of leucocytes are present in the milk from infected udders, and when their presence is noted in Breed smears, mastitis is suggested. The amount of sediment that a sample of milk contains is, of course, an index of the extent to which it has been contaminated and is often, though not necessarily always, correlated with the bacterial count.

The Hygienic Control of Milk. The sanitary quality of milk may be controlled in one or both of two ways: in the first instance, by preventing to a considerable degree the contamination of milk and the multiplication of contained bacteria; and, in the second, by destruction of bacteria, the pathogenic forms in particular, already present in the milk.

Inspection. The periodic inspection of dairy farms is carried out by the local board of health in many places, and, although it does not insure the absence of pathogenic bacteria from milk, it is effective in increasing cleanliness and reducing the numbers of bacteria. A score card is frequently used, and a given dairy farm is rated according to a system of points.

Certified Milk. One of the earliest attempts to avoid the dangers of milk-borne infection was the elaboration of methods designed to safeguard milk at every step in its production, collection and distribution. To this end "Medical Milk Commissions" were established in a number of localities in the United States, usually under the auspices of the local medical society. Milk conforming to certain standards is certified by such a commission to be of high quality. The regulations, which are generally excellent, deal with such matters as the cleanliness of barnyard and dairy; the purity of the farm water supply; the proper sterilization of utensils; and the health of the cows and of the milkers. The bacterial content is limited to 10,000 per milliliter and the milk must be delivered within thirty-six hours.

Certified milk is undoubtedly safer to use than milk collected and transported without suitable supervision, and the work of the milk commissions has done much to improve dairy conditions in many parts of the country. At the same time, raw milk, certified or not, can never be regarded as protected against all chances of contamination; the difficulty—not to say impossibility—of making sure that no typhoid carriers and no persons suffering from a mild case of diphtheria or scarlet fever are ever employed in a dairy is, of course, self-evident. Outbreaks of diphtheria, paratyphoid fever and other diseases have, in fact, been traced to certified milk. For this reason, as well as because of the relatively high cost of production, the use of certified milk remains limited.

Pasteurization. The process of destroying pathogenic bacteria in milk is by far the most satisfactory method of controlling milk-borne infection. The use of a temperature high enough to kill most microorganisms but not so high as to produce radical alterations in the substance heated was first applied by Pasteur to preserving wines without destroying their original flavor or bouquet. Al-

though still widely used in connection with bottled beer and wines, the process of pasteurization is now used chiefly for the treatment of milk.

The temperature to which milk is raised and the time for which it is held there are, of course, dictated by the heat resistance of the bacterium to be killed. From the beginning attention has been directed primarily toward the tubercle bacillus, and it has been found that this bacterium is killed by exposure to a temperature of 140° F. for twenty minutes. In practice, then, a temperature of 142° to 145° F. for a period of thirty minutes provides an adequate margin of safety, and these are the requirements that are usually specified.[5] It should be noted that the technical aspects of such treatment of milk on a large scale are of prime importance, taking the form of prevention of foaming, proper design of valves to prevent "cool pockets" and dead ends, and the like. It might be supposed that a higher temperature or longer holding time resulting in a greater margin of safety would be desirable; this is, however, not the case, for if either the time or the temperature is increased alterations take place in the physical state of the milk in which the fat is dispersed into smaller globules and will not rise to the top on standing. Such a disturbance of the "cream line" is, of course, of esthetic rather than sanitary significance.

Considerably higher temperatures and a short holding time have been and still are used to a limited extent. In the so-called "flash" process the temperature is raised to 160° F. and maintained for fifteen seconds, then the milk is immediately chilled, as it is in the "holding" process discussed above. Both time and temperature are difficult to control accurately in the flash process, and this fact, together with the "cooked" taste imparted to the milk, has severely limited the use of this method.

The plate count of milk is tremendously reduced by pasteurization, for not only are the pathogenic bacteria, such as the tubercle bacillus, *Brucella abortus*, streptococci and the like, destroyed, but the majority of other bacteria present as vegetative cells are killed also. The efficiency of the process may, then, be measured in terms of bacterial destruction and, in the last analysis, must be measured this way. Very recently, however, a test has been devised which apparently gives an accurate measure of the efficiency of pasteurization as carried out in practice. The *phosphatase test* is based upon the presence of the heat-sensitive enzyme phosphatase in milk. Since 96 per cent of the enzyme is destroyed by heating to 143° F. for thirty minutes, the amount remaining in a pasteurized milk may be used as an indicator of the pasteurization as carried out. The enzyme liberates phenol from phosphoric-phenyl esters and, as originally proposed, the test consisted of the addition of disodium phenol phosphate and Folin's reagent, incubating eighteen to twenty-four hours, and reading the blue color developed. A variety of modifications has, however, been proposed, and as yet the test has not been standardized,[6] though it has become increasingly widely used.[7]

Not all bacteria are killed by pasteurization. Besides the resistant spore formers, aerobic and anaerobic, certain streptococci are able to survive the pas-

[5] Amer. Jour. Hyg., 1927, 7:147.
[6] For discussion and references see the appendix of *Standard Methods for the Examination of Dairy Products, loc. cit.*
[7] Burgwald: Jour. Dairy Sci., 1942, 25:285.

teurizing temperature. These are the lactic acid formers rather than the pathogenic forms, and pasteurized milk sours in the ordinary way on standing, though a longer period of time is required—*i.e.*, its keeping qualities are improved. If higher temperatures are used, 180° F., the lactic acid bacteria are killed and proteolysis occurs as the milk spoils. Thermophilic bacteria are frequently present in pasteurized milk in great numbers, for the temperature of pasteurization is an incubation temperature for these microorganisms. The slight acid metallic taste occasionally noticeable in pasteurized milk is often attributable to their biochemical activity.

It must be emphasized that the process of pasteurization, while effective in destroying pathogenic bacteria present in milk and, incidentally, increasing its keeping qualities, is not to be regarded as an excuse for the marketing of dirty and highly contaminated milk. There is some evidence, for example, that the growth of enormous numbers of bacteria, albeit nonpathogens in the usual sense, is associated with summer diarrhea of infants. In most cases, therefore, sanitary regulations specify not only the allowable number of bacteria in pasteurized milk as delivered but also an upper limit for raw milk which is to be pasteurized.

The Regulation of Milk Quality. The application of appropriate methods of rendering and keeping milk satisfactory from the hygienic point of view is, essentially, a social and legal problem rather than a scientific one. To this end appropriate ordinances are more and more generally incorporated into the legal structure of the community, and these, when adequately enforced, produce a marked reduction in milk-borne disease. A standard form of ordinance developed by the United States Public Health Service[8] had in 1938 been adopted by more than 800 American cities. In any case, the grading and pasteurizing of milk has become general in the United States. A survey[9] has shown that about half the cities of over 1000 population grade milk and permit the sale of one grade of raw milk and one grade of pasteurized milk; of the total volume of market milk about 74 per cent is pasteurized, 99.4 per cent is from tuberculin-tested herds, and 35 per cent from abortion-tested herds. In general, sanitary regulations are somewhat more strict and more rigorously enforced in the large cities. In Chicago, for example, all milk is pasteurized, including certified milk, and this regulation is rigidly enforced.

Such practice has resulted in marked reductions in the number of bacteria in market milk. In 1901 the bacterial content of market milk in New York City varied from 300,000 in the coldest weather to 5,000,000 during the summer months; in Chicago (1904) the counts ranged from 10,000 to 74,000,000, and in Boston (1892) averaged 4,500,000. The incidence of milk-borne disease has correspondingly decreased; for example, in 1907–1915 there were in Massachusetts 2215 cases of typhoid fever which were traced to milk, but in 1919–23 only 297.

Milk Products. Various foods made from milk, such as ice cream, butter, cheese and the like, are potential vectors of disease when made from milk contaminated with pathogenic bacteria. The bacteria tend to die out upon stor-

---

[8] Pub. Health Repts., 1926, *41*:1604.

[9] Fuchs and Frank: Pub. Health Bull. No. 245, 1938; also Pub. Health Repts., 1942, *57*:228.

age, of course, although it has been found that the typhoid bacillus will survive for three months or more in butter, and tubercle bacilli have been found in butter and certain quick-ripening varieties of cheese. Ice cream may serve as a vector for typhoid fever, scarlet fever and the like. Pasteurization of the mix is customary and generally at higher temperatures than those used for milk. Human infections with foot-and-mouth disease have been traced to contaminated butter and cheese, but the public health significance of these findings is problematical.

<div align="center">

FOOD POISONING AND FOOD-BORNE INFECTION[10]

</div>

The diseases transmitted by milk and milk products may be disseminated by a variety of other foods; in addition to these, however, other types of illness may result from the ingestion of contaminated foods which make up that group of affections designated as food poisoning. The kinds of illness that may result from the ingestion of food may be summarized briefly:

   (1) individual idiosyncrasies;
   (2) toxemia from foods, such as
      (a) foods naturally poisonous,
      (b) foods into which poisons have been accidentally introduced and
      (c) foods containing poisons of bacterial origin formed by
         1. *Clostridium botulinum* and
         2. staphylococci;
   (3) food-borne infection, including both
      (a) bacterial infections, such as
         1. typhoid fever, dysentery, cholera *et al.* and
         2. Salmonella infection, and
      (b) parasitic infections.

It will be clear that in some instances food serves simply as a vector in the transmission of diseases such as the parasitic infections and the enteric diseases. In the remainder, however, the clinical manifestations are those associated with food poisoning proper—vomiting, diarrhea, enteritis and a greater or lesser degree of prostration. Although a number of types of food poisoning given above are not bacterial in origin, their clinical symptoms frequently are those of bacterial food poisoning, and these must be considered in any attempt to ascertain the etiology of a given outbreak, even though food poisoning is, in a majority of instances, a consequence of bacterial activity. Hypersensitivity (p. 334) to a given food substance, for example, is frequently manifested as vomiting, and an outbreak confined to a family may be the result of familial tendency; similarly, the gastro-intestinal disturbances following the ingestion of naturally poisonous foods such as toadstools, or foods contaminated with poisons such as arsenic or cyanide, are often indistinguishable from those induced by some poisons of bacterial origin.

Food Poisons of Bacterial Origin. Often popularly termed "ptomaine poisoning," poisoning with food containing toxic substances of bacterial origin is very common, probably more so than is generally recognized. The term "ptomaine poisoning" is a misnomer that is both misleading and inaccurate.

[10] Jordan: *Food Poisoning and Food-Borne Infection.* 2nd ed. University of Chicago Press, Chicago. 1931. Dack: *Food Poisoning.* University of Chicago Press, Chicago. 1943.

The organic bases such as putrescine, cadaverine, methylamine and the like, which have been called ptomaines and which result from the bacterial decomposition of protein, are not toxic when given by mouth. Neither are other decomposition products toxic *per os*. While a partially decomposed food may be esthetically unattractive, the innocuous nature of the products of decomposition is obvious when one considers the advanced state of decomposition reached by some cheeses. Toxicity is, on the contrary, attributable to the presence of substances synthesized by the bacteria whose presence may or may not be associated with obvious evidence of decomposition of the food substance.

Botulism. Of the toxic substances formed by bacteria in food the most powerful is the toxin of *Clostridium botulinum* (p. 593). In the United States canned foods provide the anaerobic conditions necessary for the germination of botulinus spores which have survived processing, while in Europe meat products, particularly the larger sausages, have been more frequently involved. The microorganism never infects man under natural conditions, and the disease is an intoxication resulting from the ingestion of preformed toxin present in the food substance. The disease is not of great public health importance—about 2000 cases have been reported in all—and the outbreaks are generally limited in scope, often being confined to a family circle of a few individuals. The case fatality is high, however, probably 60 to 80 per cent, and when outbreaks do occur they attract considerable attention. This type of food poisoning differs from the others in that the symptoms are not necessarily gastro-intestinal; the toxin acts upon the peripheral nerves and the symptoms are those resulting from such damage. The use of botulinum antitoxin is discussed elsewhere.

Since both anaerobic conditions and a period of incubation are required for the growth of *Cl. botulinum* and the formation of toxin, fresh foods are not involved in this type of food poisoning. The toxin is destroyed by heat, hence freshly cooked foods do not contain it. Canned foods and certain meat products which are consumed either cold or after simply warming may contain botulinum toxin if the spores of this bacterium were originally present and not destroyed. Commercial processing as now employed in this country is adequate to destroy botulinum spores, and at the present time outbreaks of botulism are frequently attributable to the consumption of home canned, insufficiently heated foods. The presence of toxin is not necessarily associated with obvious signs of spoilage, though it is probable that toxin-containing foods are never entirely normal in appearance, odor and the like.

Staphylococcus Food Poisoning. The marked gastro-intestinal disturbances characteristic of food poisoning are not infrequently associated with the consumption of foods containing starch thickening, such as eclairs, cream puffs, certain types of cake fillings and salad dressings, and the like. Upon bacteriological examination, the incriminated food generally is found to contain enormous numbers of bacteria, sometimes staphylococci, other times bacteria of the colon-aerogenes group, streptococci, Proteus and similar microorganisms.

The causal relation of staphylococci, generally, though not necessarily, hemolytic strains of the *Staphyloeoccus aureus* group, to food poisoning was

suggested in 1914[11] and in recent years this relationship has been definitely established.[12] These bacteria produce a soluble toxic substance, or *enterotoxin*, which gives rise to typical food poisoning symptoms in man and rhesus monkeys upon feeding and in kittens upon injection. There is considerable doubt as to the specificity of the kitten test, however, for it has been shown that the staphylolysins will cause vomiting on injection into these animals.[13] The incubation period in man is short—two to six hours— and the case fatality *nil*, with complete recovery in twenty-four to forty-eight hours. The nature of the enterotoxic substance is unknown, although its formation by staphylococci appears to be favored by the presence of starch. A period of incubation is necessary for its elaboration by the bacteria, and in outbreaks of this type of food poisoning it is always found that a period of time, generally not less than eight hours, has elapsed between the preparation and the consumption of the food. This type of food poisoning is quite common and a number of well authenticated outbreaks have been reported.[14]

Whether or not bacteria other than the staphylococci form similar enterotoxic substances is open to question. The fact that the same incubation period and clinical symptoms are observed when epidemiological evidence incriminates the other bacteria noted above suggests that enterotoxin may be formed by them. Streptococci are occasionally incriminated in food poisoning outbreaks with evidence of enterotoxin formation,[15] and *Bacterium aerogenes*, Proteus and similar organisms have been found under much the same circumstances. There is some experimental evidence[16] indicating that the ability to form substances irritating to the human alimentary tract may be one that is possessed by a variety of bacteria, but as yet this is not definitely established.

Food-Borne Bacterial Infections. The food-borne bacteria infections are of two general types. The one consists of those diseases which are transmitted by a variety of vectors of which food is but one and whose clinical symptoms are not those usually associated with food poisoning. Such, for example, are typhoid and paratyphoid fevers, dysentery, cholera and other enteric infections. The second type of infection is that with bacteria of the Salmonella group, in which the incubation period is short, the gastro-intestinal disturbance is of short duration, a day or two, and the symptoms are typical of food poisoning. There is some crossing between the two types, for paratyphoid B bacilli (*Salmonella paratyphi* B) may produce either the typical food poisoning response or paratyphoid fever.

Food-borne typhoid, dysentery and other diseases of the first group ordinarily occur on a small scale; the great epidemics are water-borne and, to a lesser extent, milk-borne. Although limited in scope, food-borne infection plays an important part in maintaining some of these diseases in endemic form. The so-called "residual typhoid," for example, which remains in spite of hygienic

[11] Barber: Philippine Jour. Sci., 1914, 9:515.
[12] Dack, Cary, Woolpert and Wiggers: Jour. Prev. Med., 1930, 4:167; Jordan: Jour. Amer. Med. Assn., 1930, 94:1648.
[13] Fulton: Brit. Jour. Exp. Path., 1943, 24:65.
[14] *Cf.* Jordan and Burrows: Amer. Jour. Hyg., 1934, 20:604.
[15] Foley, Wheeler and Getting: Amer. Jour. Hyg., 1943, 38:250.
[16] Jordan and Burrows: Jour. Inf. Dis., 1935, 57:121.

control of water and milk supplies is, in large part, food-borne. The employ-
ment of cooks and other food handlers who are typhoid carriers provides the
opportunity for food infection and consequent transmission of the disease.
Mary Mallon—"Typhoid Mary"—was one of the most notorious examples of
a cook who was a typhoid carrier.

Of the Salmonella infections, one of three species is generally involved, *Sal-
monella typhi-murium* (*S. aertrycke*) and its varieties (*newport, stanley,* etc.),
Salmonella enteritidis and *Salmonella cholerae-suis* (including Voldagsen and
paratyphi C). *S. typhi-murium* is the most frequently observed, while *S.
cholerae-suis* is only rarely present. The ingestion of food containing large
numbers of these organisms frequently results in the typical symptoms of
food poisoning. No enterotoxic substance has been shown to be formed by
these bacteria, and it is probable that actual infection takes place as indicated
by the somewhat longer incubation period and the finding of the bacteria in
the feces. The case fatality is variable, ranging from zero to 10 per cent. Almost
any kind of food may serve to carry these microorganisms, although meats and
other protein foods predominate. This type of food poisoning is not common.[17]

Both *S. typhi-murium* and *S. enteritidis* are commonly carried by rats and
mice, and it is probable that in many cases these rodents are responsible for
the infection of food. In others the source of infection may be a human
carrier, and in many cases of meat poisoning it has been found that the meat
was from a diseased animal.

Shellfish and Disease. Shellfish, including oysters, clams and mussels,
have been responsible for the transmission of enteric disease, especially typhoid
fever, through pollution of areas in which they are grown or stored. They are
commonly eaten in an uncooked or partially cooked condition which facilitates
transmission of disease; it has been found that, while scalloped oysters, fried
clams and clams in chowder are practically sterilized, steamed clams, fried
oysters, oysters in stew and mussels cooked in the usual ways are not freed
from coliform bacteria. The shellfish, in the course of breathing and feeding,
filter large quantities of water and readily take up enteric pathogens from pol-
luted beds or when placed in brackish waters near sewage outfalls to "fatten."
Imbibed bacteria, if not ejected very soon, are passed through the gastro-in-
testinal tract and discharged in about five hours. In the warm season the pollu-
tion of the water of a bed or storage area is highly correlated with infection of
the shellfish, but during the cold months the organisms commonly show little or
no infection, presumably because of greatly reduced metabolic activity. The
rapidity with which bacteria are passed through shellfish suggests the possi-
bility that they may be cleansed of infection by storage in clean or even chlo-
rinated water. The possibility is not only feasible but practiced, four days
sufficing to practically eliminate all coliform bacteria. Essentially the same
methods that are used for the bacteriological examination of water (p. 253)
are applied to shellfish. The United States Public Health Service has suggested
that not more than 50 per cent of the 1 ml. samples of pooled shell liquor and
finely chopped tissue of 10 or more oysters, clams or mussels should show

[17] Savage and White: *Food Poisoning: a Study of 100 Recent Outbreaks.* Medical Re-
search Council Special Report Series No. 92, 1925.

coliform bacteria in the presumptive test, but the figure is a guide rather than a standard and is not inflexible.

The food-borne parasitic infections include the various flukes, tapeworms, echinococcus and round worms that infect man. In these diseases the infective stage of the parasite is often present in a food substance as a consequence of its life cycle and the mechanisms involved may be found elsewhere. (See Chapter 34.)

IMMUNITY—ANTIGENS, ANTIBODIES AND THE ANTIGEN-ANTIBODY REACTION[1]

It has been common knowledge for many years that recovery from certain of the infectious diseases is accompanied by the development of an enhanced resistance, and second attacks of a disease once overcome are not common. This enhanced resistance, or immunity, is specific, *i.e.*, an individual immune to one disease may be no more than ordinarily resistant to others, and is variable from one disease to another, some giving rise to a "solid" immunity of long duration while in others the immunity is imperfect or partial and transient. The specific immune state supplements the complex of factors which make up non-specific resistance to infection, and in some instances even a solid immunity may be broken down by fatigue, malnutrition and similar factors which are not consistent with a state of physiological well-being. Immunity arises as a consequence of the reaction of the host to intimate contact with the parasite, or its products, within the tissues, and constitutes a last line of defense whose presence frequently prevents infection and whose development during an attack of disease determines the outcome. The reaction of the host to the invading microorganism has been studied intensively since the early days of bacteriology, and the body of knowledge so accumulated makes up the extraordinarily complex science of immunology. These studies have not only led to some degree of understanding of the phenomenon of specific resistance to infection but in addition have provided biology with a new method whose general application has been, thus far, limited.

ANTIGENS

An antigen is ordinarily but unsatisfactorily defined as any substance whose introduction into the tissues of an animal results in the appearance, after a suitable length of time, of antibodies in the blood serum and other body

[1] Much of the earlier work on immunity and related problems will be found in the following volumes: Ehrlich: *Gesammelte Arbeiten zur Immunitätsforschung*. Berlin. 1904; Metchnikoff: *Leçons sur l'Inflammation*. Paris. 1892; *Idem: L'Immunité dans les Maladies Infectieuses*. Paris. 1901; Bordet: *L'Immunité*. Paris. 1920. The following modern treatises are of particular value: Zinsser, Enders and Fothergill: *Immunity. Principles and Application in Medicine and Public Health*. 5th ed. The Macmillan Company, New York. 1939; Wells: *Chemical Aspects of Immunity*. 2nd ed. Chemical Catalog Co., New York. 1929; Topley: *An Outline of Immunity*. William Wood & Company, Baltimore. 1933; Medical Research Council (Great Britain): *A System of Bacteriology*. Vol. 6; Marrack: *The Chemistry of Antigens and Antibodies*, Medical Research Council, Special Report Series No. 230, 1939. Boyd: *Fundamentals of Immunology*. 2nd ed. Interscience Publishers, New York. 1947.

fluids. With certain exceptions, the reaction is *specific* in that each antigen stimulates the formation of antibody for itself and no other antigen, and it takes place only when the antigen is a *foreign substance* to the animal into which it is injected. This characterization is, of course, one that is based on what antigens do rather than what they are, for knowledge of the nature of these substances is as yet too fragmentary to permit a general definition in terms of composition and configuration of the chemical compounds which exhibit the property of antigenicity.

In general, antigenic substances are proteins, and probably all naturally occurring proteins soluble in the body fluids and containing a full complement of amino acids (the so-called complete proteins) may function as antigens. According to Wells[2] the presence of aromatic radicals is associated with antigenicity; proteins deficient in aliphatic amino acids but containing aromatic amino acids, such as zein, gliadin, egg albumin and casein, are antigenic, while gelatin and protamines which are deficient in aromatic radicals are not antigenic. The property of antigenicity is not destroyed by heating except as the protein is rendered insoluble by coagulation (specificity is somewhat altered), but it is lost upon hydrolysis, probably at a very early stage, although the precise point at which antigenicity disappears during hydrolytic cleavage of the molecule is not known. Antigenicity, then, appears to be a property of the intact, or nearly intact, protein molecule, and there is reason to believe that one prerequisite of antigenicity is a large molecule which may exist in colloidal solution.

Iso-antigens. It is not strictly true that antibody response is induced only by antigens foreign to the inoculated animal unless the term foreign is taken to mean not present in the circulation of that animal. It was early observed by Hektoen[3] that thyroglobulin would act as an antigen in the same species of animal from which the tissue was taken, and since then it has been found that lactating goats will produce antibody to their own casein and that lens protein is antigenic even in the same individual animal. It has long been known, too, that the serum of the viper will protect against the venom of that snake as efficiently as the best hyperimmune horse serum. It is definitely established, therefore, that an animal will produce antibody against certain antigens occurring in its own tissues. Such antigens are designated iso-antigens and their corresponding antibodies iso-antibodies. The organ specificity of certain types of antigens (see below) is probably a related phenomenon. In general, iso-antibody titers are low.

The Human Blood Groups.[4] It was discovered by Landsteiner[5] that human blood could be divided into four immunological groups which represent the four possible combinations of two antigens present in the erythrocyte. The serum contains antibody (agglutinin) for the absent antigens; antigen and corresponding antibody do not coexist in the same blood. There are three systems of nomenclature for these groups (see table) of which the International

[2] Wells: *The Chemical Aspects of Immunity.* 2nd ed. Chemical Catalog Co., New York. 1929.

[3] *Cf.* Lewis: Jour. Inf. Dis., 1934, *55*:168.

[4] See the review by Loutit: Nature, 1944, *153*:97; and the discussion by Wiener and Karowe: Jour. Immunol., 1944, *49*:51.

[5] Landsteiner: Wien. Klin. Wchschr., 1901, *14*:1132.

is by far the most widely used. Additional immunological factors are also known to be present and are responsible for a part of the observed transfusion incompatibilities; these can be avoided if donor and recipient blood are tested against one another as well as typed. These blood groups are inherited by Mendelian law and for this reason are of considerable interest in connection with the question of normal antibodies (p. 329) as well as having some forensic utility in cases of disputed paternity, etc. Blood groups occur in lower animals as well as in man, as in the rat[6] and in cattle.[7]

The Specificity of Antigens. Closely associated with the nature of antigens is the specificity of the immunological reactions. Not only does a given antigen stimulate the formation of a specific antibody but it will react, either *in vivo* or *in vitro*, only with its own antibody or antibodies to closely related antigens. For example, the serum proteins of the higher animals, while highly specific for species, show cross reactions with the antibodies to the serum proteins of closely related species; human blood serum shows no immunological relationship to horse or rabbit serum but reacts to some degree with antisera prepared against the serum proteins of anthropoid apes and certain monkeys.[8] Immunological differences between individuals of a single species are, of course, found in the human blood groups. The inheritance of blood groups and the correspondence of immunological relationships of species with generally

HUMAN BLOOD GROUPS

| Groups | | | Erythrocyte antigens | Serum antibodies |
International	Moss	Jansky		
O	IV	I	....	a, b
A	II	II	A	b
B	III	III	B	a
AB	I	IV	A, B	....

accepted zoological classifications are indicative of the fundamental significance of antigenic specificity.[9]

It should be noted, however, that, although the great majority of antigenic substances are species-specific, certain antigens are found to occur in distantly related organisms. Of these the more important are lens protein (actually two proteins, α and β crystallin), which is common to a wide variety of animals, and an antigen known as Forssman antigen, heterophile antigen or, less frequently, heterogenetic antigen. Heterophile antigen has been found in the organs of the guinea pig, horse, cat, dog, mouse, chicken, turtle and several

[6] Burhoe: Proc. Nat. Acad. Sci., 1947, 33:102.
[7] Singh: Indian Jour. Vet. Sci. Animal Husb., 1942, 12:12.
[8] Such immunological relationships are discussed at length by Nuttall: *Blood Immunity and Blood Relationship.* Cambridge. 1904.
[9] See the review by Boyden: Physiol. Zool., 1942, 15:109.

species of fish, in some bacteria, such as certain of the paratyphoid and dysentery bacilli and pneumococci, and in some varieties of maize. It is not present in the erythrocytes of any of these animals but is present in the red cells of the sheep, whose organs do not contain it. It is not found in other organisms such as the pig, ox, rabbit, goose, frog, eel, man, pigeon and rat. This peculiar distribution among species has not been explained.

Still other antigens which are common to, or closely related in, widely different species are those which confer organ specificity. Possibly the immunologically related caseins may be considered in this category but, further, antigens present in a given organ such as kidney are similar to those in the same organ of a different species. These have been studied in some detail by Witebsky.[10] The so-called heavy proteins, isolated from disintegrated normal tissue by ultracentrifugation and thought by some to represent mitochondria, have been of interest in connection with the isolation of similar material from virus-infected tissues which is believed to represent the infectious agent. Immunological studies on these substances by Furth and Kabat,[11] and by Henle, Chambers and Groupe,[12] have indicated that heavy proteins from various organs show three kinds of immunological specificity, namely, species specificity or immunological relation to other organs of the same animal, organ specificity, or immunological relation to the same organ of other species, and, finally, specificity for the organ of the one species. Similarly, Bailey and his co-workers[13] have reported finding organ specificity in nutrient media which was derived from the tissues used for the preparation of the infusion. It has been reported by some that organ-specific antisera are specifically toxic for that organ, *i.e.*, nephrotoxic, etc., but the evidence is not altogether unequivocal.

A number of antigens are common to different species of bacteria or to bacteria and certain other organisms and will be discussed in the following chapter in connection with natural immunity.

The Chemical Basis of Specificity.[14] A large body of sound experimental evidence has established the fact that the specificity of antigens is determined by their chemical composition. Early experimental investigation of a wide variety of antigenic proteins has shown that immunologically identical proteins are, so far as can be determined, identical in composition; that antigenic proteins differing from one another in composition are also immunologically distinct; and that antigens showing some degree of cross reaction are closely related in chemical structure. Conclusive evidence that immunological specificity is a property of certain atomic and molecular arrangements has, however, been obtained through the study of altered specificity and artificial antigens.

The specificity of an antigenic protein may be altered by heating, partial denaturation, treatment with formaldehyde, etc., in such a way that part of the original specificity is lost but species specificity remains although somewhat

[10] Witebsky: Ztschr. f. Immunitätsforsch, 1929, 62:3.
[11] Furth and Kabat: Jour. Exp. Med., 1941, 74:247.
[12] Henle, Chambers and Groupe: Jour. Exp. Med., 1941, 74:495.
[13] Bailey and Gardner: Jour. Immunol., 1940, 39:543; Bailey and Raffel: Amer. Jour. Hyg., 1941, Sec. B, 33:86; *ibid.*, 1941, Sec. B, 34:8; Jour. Exp. Med., 1941, 73:617.
[14] This subject is discussed in detail by Wells (*loc. cit.*) and by Landsteiner: *The Specificity of Serological Reactions.* Charles C Thomas, Springfield, Ill. 1936.

broadened. Heated rabbit serum, for instance, behaves as a foreign protein in the rabbit, and the antibodies produced will not react with unheated rabbit serum. Retention of species specificity is indicated by the fact that antibodies to the heated serum react with the homologous antigen and to only a slight degree with heated sera from other animals. Such heated proteins are known as *coctoproteins*. Treatment of a protein with iodine, nitric acid or nitrous acid (the last to give the intermediate diazo compounds), on the other hand, alters the specificity of the antigen so profoundly that species specificity is largely destroyed. Nitrated rabbit serum, for example, acts as a foreign protein to the rabbit, and the antibodies developed will react not only with the homologous antigen but with nitrated protein derived from other animal species or even from plants. The general question of loss of species specificity by treatment of this kind has been reviewed by Henry.[15] Since iodine, nitrate and similar substances react with the protein by substitution in the benzene rings of the aromatic amino acids, these findings have been interpreted as indicating that species specificity is associated with the aromatic radicals and their arrangement within the protein molecule, a conclusion which, as will appear, is no longer justified.

Largely through the work of Landsteiner and his colleagues[14] in the preparation and study of a large series of artificial antigens, some aspects of the phenomenon of immunological specificity are now relatively well understood. It has been found that the species specificity of antigens may be altered in ways other than attacking the aromatic radicals; acetylation with acetic anhydride, esterification with ethyl alcohol and methylation with diazomethane, all changes in the salt-forming groups of the protein molecule, result in a change in specificity similar to that obtained by treatment with iodine or nitric acid. It is of particular interest that such a marked effect on specificity may be produced by the addition of the small acetyl, ethyl or methyl groups to the large protein molecule. Even phosphorylation has been found to alter the immunological properties of egg albumin.

The influence of the addition of relatively simple organic radicals on the immunological specificity of antigenic proteins has been further elucidated through the study of the immunologic behavior of a variety of compounds prepared from protein and the diazonium derivatives of a number of methyl, chlor, brom and nitro substitution products of aniline and *o-*, *m-* and *p-*aminobenzenesulfonic acid as well as the parent compounds, and others such as *p-*aminophenylarsenic acid, *o-*, *m-* and *p-*aminocinnamic acid, etc. From these and other experiments it is apparent that the *immunological behavior of antigens is specifically modified by a relatively small part of the large protein molecule, and the specificity of the antigen is determined by the chemical structure of this part, cross reactions occurring between antigens whose determinant portions are closely related aromatic groups.* The spatial arrangement in the determinative groups, as well as their nature, is reflected in immunological behavior; the position, *ortho, meta,* or *para,* of substituted acid groups in the aromatic radical produces differences in specificity, the stereoisomers of tartaric acid and *p-*aminobenzoylphenylacetic acid yield immunologically distinct antigens when coupled with protein, and the position of amino acids in peptide-

[15] Henry: Jour. Exp. Med., 1942, 76:451.

azoproteins is a factor in determining immunological specificity. If it be assumed that the amino acids comprising the peptide chains of the protein molecule are arranged in regular and recurring order and proportion and, consistent with present theory of protein structure, that these chains are folded, then it is apparent that the recurring combinations of amino acids in the folded chains will result in "nodes" of molecular structure. It is supposed that this type of molecular structure confers immunological specificity on the protein molecule that is not combined with some other type of structure.

Haptenes. The relatively simple substances which, when linked to antigenic protein, determine the immunological specificity of the complex, have been termed *haptenes* by Landsteiner. They are also known as *partial antigens* since, although in the absence of a protein carrier they will not stimulate the formation of antibody, they will react with antibody produced by the haptene-protein complex and, therefore, act as "antigens" in the test tube.

The importance of haptene-determined specificity to the understanding of the immunology of bacteria and the infectious diseases is very great indeed, for it has become increasingly apparent that much of the antigenic material present in the bacterial cell is in the form of haptene-protein complexes. It was found by Zinsser and Parker[16] in 1923 that certain materials found in bacteria and presumably non-protein in nature, although not capable of stimulating the formation of antibodies, would react with antisera *in vitro*, and these they designated as *residue antigens*. Similar substances have since been found by other workers in a wide variety of bacteria, and investigation has indicated that in many instances these substances function as haptenes, lending immunological specificity to the microorganisms containing them.

Polysaccharides. Of the residue antigens those of the pneumococcus have been the most intensively studied and for present purposes may be regarded as typical. These so-called *specific soluble substances* (SSS), which make up the capsule of the bacterium, have been found by Heidelberger, Avery, Goebel and others[17] to be polysaccharide in nature. Of the pneumococcal type-specific polysaccharides, that of Type 3 has been the most thoroughly investigated and appears to be a polymer of aldobionic acid units (in this case glucose and glucuronic acid joined through a glucoside linkage, 4-β-glucuronosido-glucose, designated as cellobiuronic acid) having a molecular weight of 1000 to 5600. The nature of the other type-specific polysaccharides is not so clear. Type 2 polysaccharide hydrolyzes to glucose; Type 1 is possibly a trisaccharide made up of two uronic acid (in part galacturonic acid) molecules, a third molecule containing nitrogen, and acetyl groups whose presence is of considerable immunological significance; and Type 4 yields an amino sugar on hydrolysis. That such polysaccharide haptenes are not peculiar to the pneumococcus is indicated by the isolation of similar substances from a variety of microorganisms including yeasts and other fungi and certain metazoan parasites as well as many species of bacteria.

[16] Zinsser and Parker: Jour. Exp. Med., 1923, 37:275.

[17] *Cf.* Heidelberger: Chem. Rev., 1926–7, 3:403; Physiol. Rev., 1927, 7:107; Medicine, 1933, 12:279; Harvey Lectures, 1933, 28:184. See also Marrack: *The Chemistry of Antigens and Antibodies*. Medical Research Council Special Report Series No. 230, 2nd ed., 1939.

The presence of capsular polysaccharide material confers immunological specificity upon the pneumococcus types; the rough, nonencapsulated forms are species-specific and no longer may be differentiated into types irrespective of the immunological type of the smooth, encapsulated parent form. The species specificity manifested by the rough forms, furthermore, appears to be intimately associated with the presence of yet another polysaccharide, the so-called "C" polysaccharide, which is common to the rough forms and which, like Type 4 polysaccharide, hydrolyzes to an amino sugar but also contains phosphoric acid. The conversion of pneumococcus types, then, would appear to be an alteration in the ability of the microorganism to synthesize one or another of the type-specific polysaccharides (p. 182). The immunological specificity of other bacteria, including types A, B and C of Friedländer's bacillus, certain of the meningococcus types, staphylococci, streptococci and other microorganisms, is similarly associated with the presence of polysaccharide haptenes. The nature of the linkage between polysaccharide and antigenic protein in the bacterial cell is not known.

It has been shown also that an effective antigen need not contain intact polysaccharide; Goebel[18] has prepared an azoprotein antigen from horse serum and the glucoside of cellobiuronic acid whose antiserum will protect mice against Type 3 pneumococcus infection. Similar protection is afforded against Type 2 infection by an antiserum to an azoprotein antigen prepared from the glucoside of a *synthetic* isomeric aldobionic acid, gentio-biuronic acid ($6\text{-}\beta$-glucuronosido-glucose).

Lipids.[19] Principally because of the apparent solubility of Forssman antigen in organic solvents and the immunological activity of certain lipoidal preparations from tubercle bacilli it has been thought that lipids might function either as true antigens (see below) or as haptenes. The experimental evidence, *e.g.*, immunization with mixtures of foreign serum and such substances as lecithin, cephalin and cholesterol, has, however, been inconclusive. It is, therefore, uncertain as yet whether or not lipids may determine the immunological specificity of antigenic proteins. In this connection it is of some interest that the Forssman antigen, generally regarded as the best example of the immunological activity of lipids, contains polysaccharide haptene. In higher animals the polysaccharide-protein antigen contains lipoidal substances, but these are lacking in heterophile antigen of bacterial origin.

Non-protein Antigens. Although in general only proteins and protein-haptene complexes will stimulate the formation of antibodies, there is some evidence that certain non-protein substances may act as true antigens. Such evidence is, however, difficult to interpret in many instances because of the minute amounts of antigenic protein that are required to elicit an immunological response—according to Wells[2] as little as 0.00005 milligram of crystalline egg albumin is effective—which may be present as contamination in naturally occurring non-protein substances. A second possibility that is difficult to eliminate is that of the combination of a non-protein substance with body protein of the animal into which it is injected, leading to the formation of an

---

[18] Goebel: Jour. Exp. Med., 1939, 69:353. *Ibid*, 1940, 72:33.

[19] The question of the antigenicity of lipids is reviewed by Weil: Bact. Rev., 1941, 5:293.

antigenic complex. It is well known that such simple compounds as formaldehyde will act in this manner, and drug idiosyncrasies are not infrequently accounted for on this basis.

Glucosides. It was early reported by Ford that the glucoside hemolysin present in the toadstool (*Amanita phalloides*) and the toxin of poison ivy (*Rhus toxicodendron*),[20] also a glucoside, would, upon injection, stimulate the production of antibodies. Boivin and Mesrobeanu[21] have shown that gluco-lipids, consisting of a complex polysaccharide linked to a phosphatide or nucleotide component, present in certain of the colon-typhoid-dysentery group of bacteria and apparently representing the endotoxin of these organisms, are antigenic and give rise to antitoxic sera when injected into experimental animals. Evidence such as this suggests that under certain circumstances such non-protein substances may function as true antigens although they appear to be "poor" antigens in that the antibodies produced are generally present only in low titer.

Polysaccharides. Considerable interest has attached to the possibility that the high molecular weight polysaccharide haptenes may act as antigens apart from the protein molecules to which they lend immunological specificity. Although earlier studies indicated that pure polysaccharides, those of the pneumococcus types, functioned only as haptenes, more recent work has suggested that these substances may be complete antigens and capable of inducing an immunological response. Their immunological behavior is peculiar in some respects, but it appears definitely established that these substances will stimulate the formation of antibodies. The antigenicity of other polysaccharides, such as the vegetable starches, is not established by unequivocal experiment, and for the present these substances may be regarded as not antigenic.

Lipids. As indicated above, lipids apparently do not function as haptenes and, as might be inferred, cannot function as antigens. The antigenic activity of ethereal extracts of tissues containing Forssman antigen and that of similar preparations from the tubercle bacillus and other acid-fast bacteria may be accounted for on the basis of protein impurities. More specifically, the lipid portion of the somatic antigen of the dysentery bacilli does not appear to contribute to its antigenicity or specificity.[22]

The Antigenic Structure of Bacteria. It is already apparent that the cell substance of a bacterium is not immunologically homogeneous but consists of a number of different antigenic components. The particular combination of antigens, one or more of which may be shared by closely related forms, that goes to make up the immunological character of a bacterial species has been aptly termed an *antigenic mosaic.* This antigenic mosaic is undoubtedly an expression of deep-seated characters that contribute to the biological individuality of the bacterial species, for, from the biological point of view, these antigenic substances are qualitatively different kinds of protoplasm. In this light the immunological method may be regarded as an analytical method, remarkably sensitive, highly specific and crudely quantitative.

In a number of cases these antigens have been separated by biochemical

[20] Ford: Jour. Inf. Dis., 1906, 3:191; *ibid.*, 1907, 4:541; Jour. Pharmacol., 1910, 2:145.
[21] Boivin and Mesrobeanu: Rev. Immunol., 1935, 1:553; *ibid.*, 1936, 2:113; *ibid.*, 1937, 3:319; Ann. Inst. Pasteur, 1938. 61:426.
[22] Partridge and Morgan: Brit. Jour. Exp. Path., 1940, 21:180.

11

methods and something of their nature is known. The polysaccharide haptenes referred to above are of very considerable immunological importance in many, but not all, cases. The isolation of such substances is relatively simple, usually involving a primary alcohol precipitation from solution of the bacterial cell substance. The somatic antigens of the enteric bacilli, which appear to be identical with endotoxin in many cases, may be prepared by extraction of the intact cells with M/2 trichloracetic acid in the cold,[23] by extraction of the intact cells with glycols such as diethylene glucol,[24] by fractionation of tryptic digests of the cells,[25] or by dissociation and extraction in 6 M urea.[26] These substances may be precipitated from the crude extract by alcohol and further purified. Though it has been indicated earlier that these substances are polysaccharide-lipid complexes, there is not complete agreement on this point. Preparations made by primary trichloracetic acid extraction do appear to be of this nature but those isolated by glycol extraction are polysaccharide-lipid-polypeptide complexes. These may be dissociated and reconstituted in hot formamide solution and the polypeptide portion seems to be essential to antigenicity. Sharp separation of the immunological specificity of the flagellar antigens has not been possible by chemical means, but physical separation in which the flagella are broken off by shaking and separated from the cell proper by differential centrifugation has been accomplished. So far as is known, the flagellar antigens are protein in nature. In the past a good deal of work has been done with "nucleoprotein" separated from the bacterial cells by extraction with dilute alkali. The immunological entities have not been separated in such preparations, however.

The number of antigens that can enter into this mosaic in a single species is unknown; some Salmonella species have been found to contain seven or eight separate antigens which differ from one another not only immunologically but also in their resistance to heat, alcohol and the like, and these and other bacteria will undoubtedly prove to be increasingly complex with further study. The number of antigens demonstrable by antigenic analysis (p. 300) is, of course, only a minimum since a given antigen remains an entity only so long as possible components of it have not been found to occur separately. It is of some interest that antigens co-existing in the same cell may not be equally demonstrable. The species specificity observable in rough pneumococci, for example, is present in the smooth forms but is masked by the predominant type-specific antigens. Similar phenomena have been observed in a variety of bacterial species. Conversion to varying degrees of roughness may bring to light "new" antigens which, though presumably constantly present, have been masked by the presence of other antigenic substances. There is a tendency to interpret such findings as indicating the relative position of antigenic substances within the cell, the peeling off of "outer" antigenic layers and exposure of secondary and tertiary layers of antigen within the cell. While there is something to be said for this concept as a figurative mode of expression, it may be pointed out that, aside from the fact that in some bacteria specific antigen is

[23] Boivin and Mesrobeanu: Rev. Immunol., 1935, *1*:553.
[24] Morgan: Biochem. Jour., 1937, *31*:2003.
[25] Raistrick and Topley: Brit. Jour. Exp. Path., 1934, *15*:113.
[26] Walker: Biochem. Jour., 1940, *34*:325.

present in the capsule or flagella, there is no evidence of an immunological geography in the bacterial cell.

The demonstration of the occurrence of common antigens in heterologous bacteria may be interpreted in two ways. Thus it may be assumed that the identical antigen, or at least its determinant portion, is shared. This is a common view and one for which there is a good deal of justification in many instances. It is believed by some, however, that the observed cross reactions are attributable to the occurrence of similar determinant groups and the type of partial antigen-antibody reaction observed with artificial antigens. As yet there is no definitive evidence to substantiate either view.

ANTIBODIES

The immunological response of an animal to the initial injection of an antigenic substance is not immediate but after a suitable time interval or incubation period is manifested as an alteration in the properties of the blood serum with respect to the antigen. An immune serum, or antiserum, differs from normal serum in that it reacts, either *in vivo* or *in vitro*, with the homologous antigen. This property of immune serum is a consequence of the presence of *antibodies,* substances which are formed by the animal body in response to the presence of antigen in the tissues, and which combine specifically with the antigen. The antigen-antibody reaction is demonstrable in a number of ways, the particular technique employed depending upon the nature of the antigen. By such means five apparently different kinds of antibodies may be found, although, as will appear, these are probably but a single substance. They are as follows:

(1) the *antitoxins*—antibodies formed in response to the injection of toxins which, when mixed with the homologous toxin, neutralize its poisonous qualities;

(2) the *lysins*—antibodies which bring about a dissolution or lysis of bacterial and other cells;

(3) the *opsonins*—antibodies which sensitize bacterial cells in such a way that they are readily engulfed by the phagocytic cells;

(4) the *agglutinins*—antibodies formed in response to the injection of bacterial cell substance which, when mixed with the homologous microorganism, immobilize the bacteria if they are motile, then aggregate, or agglutinate, the cells with the formation of clumps which settle out of suspension;

(5) the *precipitins*—antibodies formed in response to the injection of antigens which, when mixed with soluble antigen, aggregate the molecules with the formation of a precipitate.

In addition to these five generally recognized kinds of antibodies, two others may be provisionally added:

(6) *ablastins*—reproduction-inhibiting antibodies which prevent the multiplication (cell division) of the invading microorganism;

(7) *neutralizing antibodies*—antibodies which, when mixed and incubated with the infectious agent, generally a filterable virus, render it noninfective.

The various antibodies may be considered briefly one by one.

Antitoxins. It was found by von Behring and Kitasato[27] in 1890 that
the immunity of rabbits and mice which had been immunized against tetanus
was associated with the ability of the blood serum to neutralize the toxic sub-
stances produced by the tetanus bacillus. The substance in the serum which
neutralized the tetanus toxin was designated by these workers as *antitoxin*.
Subsequent investigation has shown that the animal body forms antitoxins
in response to the injection of a variety of antigenic poisons, not only those
of bacterial origin such as diphtheria toxin, botulinus toxin and the like, but
also against the phytotoxins and zootoxins (p. 206).

The action of antitoxin may be directly demonstrated in the following way:
if a fatal, or many times fatal, dose of toxin be mixed with an appropriate
amount of antitoxic serum *in vitro*, the injection of the mixture into a suscepti-
ble animal is wholly without injurious effect; the poisonous qualities of the
toxin are nullified by the immune serum. The reaction is, like all other im-
munological reactions, highly specific, and an antitoxin which neutralizes
the homologous toxin is without effect on heterologous toxins. The nature of
the effect of the antitoxin which renders a powerful toxin pharmacologically
inert is unknown.

The Toxin-Antitoxin Reaction. The combination of toxin and antitoxin does
not necessitate the complete destruction of either component; neutral mixtures
of toxin and antitoxin may be dissociated by treatment with hydrochloric acid,
by freezing in the presence of phenol or tricresol and, to some extent, by simple
dilution. In certain cases, *e.g.*, pyocyaneus toxin and certain snake venoms, in
which the toxin is more resistant to heat than the antitoxin, the latter may be
selectively destroyed and the neutral mixture becomes toxic upon judicious
heating. It appears, therefore, that a more or less loose combination of toxin
and antitoxin takes place, the poisonous properties of the toxin being held in
abeyance as long as the union persists. The rate of reaction between toxin
and antitoxin, like the chemical reactions, is dependent upon temperature,
concentration, character of the medium in which the reaction occurs, and sim-
ilar factors. The avidity of an antitoxin for its corresponding toxin differs in
different cases; the union between tetanus toxin and antitoxin, for example,
take places less rapidly than that between diphtheria toxin and antitoxin.

An understanding of the precise character of the toxin-antitoxin reaction is
dependent upon the interpretation of phenomena revealed by quantitative
studies. It might be expected that a given quantity of antitoxin would always
neutralize a constant amount of toxin, that the neutralization would follow the
law of multiple proportions. This is, however, not the case, and it appears that
the amount of antitoxin required to neutralize a given quantity of toxin is
dependent upon (*a*) the manner in which the two are mixed with one another
and (*b*) the relation between toxicity and combining power in the particular
filtrate under consideration.

In the first instance it has been observed that when an excess of toxin
is added to its specific antitoxin in several portions at proper intervals of time,
much more unneutralized toxin remains in the mixture than if the same quan-
tity of toxin had been added to the same quantity of antitoxin at one time. This
is known as the *Danysz phenomenon*. If, on the other hand, antitoxin be

[27] von Behring and Kitasato: Deut. med. Wchnschr., 1890, 16:1113.

added to toxin in successive equal portions, it may be shown that, in general, the first portion of antitoxin neutralizes a greater portion of the toxin than the second, the second a greater than the third, etc. In the second case it has been found that there is no constant relationship between toxicity and combining power of a toxic filtrate; toxicity slowly diminishes upon storage but combining power remains unchanged. A similar change may be brought about by various physical and chemical agents, notably formaldehyde, which destroys toxicity but does not affect the antitoxin combining power of the preparation. Such altered toxin, which retains its antigenicity unchanged in addition to its combining powers, is designated as *toxoid* and, when prepared by treatment with formaldehyde, *formol toxoid* or *anatoxin.* The proportions of toxin and toxoid in a given filtrate are variable. Consequently there is no fixed relation between toxicity and power to combine with antitoxin; the MLD's neutralized by a unit quantity of antitoxin may vary from one filtrate to another or in the same filtrate at different times from 30 to 130.

Three theoretical explanations of the toxin-antitoxin reaction have been advanced. They are known by the names of their proponents, Ehrlich, Arrhenius and Madsen, and Bordet and Landsteiner.

According to Ehrlich the reaction is purely chemical and essentially similar to the neutralization of a strong acid by a strong base. The combining properties of toxin and antitoxin would, therefore, be a manifestation of primary valencies. This concept is, however, not compatible with the observed behavior of toxin and antitoxin in mixture; the Danysz phenomenon, for instance, suggests that if the neutralization proceeds according to Ehrlich, the reacting substances are not homogeneous but consist of mixtures of substances with varying affinities for one another. On the basis of his studies on diphtheria toxin, Ehrlich postulated a series of components of toxin which differed from one another both in their avidity for antitoxin and in their toxicity.

Arrhenius and Madsen preferred to regard the toxin-antitoxin combination as essentially a chemical reaction but analogous to the neutralization of a weak acid by a weak base (ammonia and boric acid). Although this hypothesis is attractive in many respects, it is not compatible with certain aspects of the toxin-antitoxin reaction. Simple dilution of a neutral mixture, for example, does not result in the degree of dissociation that this theory calls for. As a consequence of this and certain other discrepancies, the theory of Arrhenius and Madsen, is, like the theory of Ehrlich, not generally accepted at the present time.

A third concept, proposed by Bordet and strongly supported by Landsteiner, is that of the toxin-antitoxin reaction as an adsorption phenomenon, essentially physico-chemical in nature and arising as a consequence of intermolecular forces (secondary valencies). The varying degrees of toxicity apparent in the neutralization of toxin may be regarded as due to differences in completion of saturation of the individual toxic units, a process that may be compared to certain staining reactions such as the action of iodine upon starch, a dilute iodine solution producing a light blue tinge, a stronger solution a deep blue. This adsorption theory would view the action of antitoxin upon toxin as a sort of progressive attenuation, proportional to the amount of antitoxin added. The evidence for such a mechanism, not only in the toxin-

antitoxin reaction but in antigen-antibody reactions in general, is very strong indeed, and it is generally regarded as highly probable that these reactions are essentially physico-chemical in nature. This view of the immunological reactions will be considered at greater length in a later section.

The Standardization of Antitoxins. The quantitative evaluation of the toxin-neutralizing capacity of antitoxic sera is clearly a matter of considerable practical as well as theoretical importance. Ehrlich originally proposed as the standard unit of diphtheria antitoxin that amount which would just neutralize 100 guinea pig MLD's of toxin. As indicated above, however, variability in the relation of toxicity to combining power invalidates any standard based on toxicity; in other words, the combining power of a toxin is not a measure of its toxic qualities. On the other hand, since the combining power of a toxic filtrate remains constant within narrow limits, it is possible to establish an arbitrary standard unit upon which the relative strength of all antitoxic sera can be based. Such a standard diphtheria antitoxin was first prepared by Ehrlich and was preserved by him with all precautions against possible deterioration. A standard antitoxic serum based on Ehrlich's arbitrary standard unit is also prepared in this country by the National Institute of Health of the United States Public Health Service, and is distributed every two months to the licensed producers of commercial serum. The unit is international, standard sera being tested from time to time by the Biological Standardization Commission of the League of Nations.[28]

Three methods are used in the titration of diphtheria toxin and antitoxin. The first of these is the classic method of Ehrlich in which two limits (Lat., *limes*) are determined by guinea pig inoculation. These are the L_0 dose of toxin, which is defined as that amount exactly neutralized by the standard unit of antitoxin, and the L+ dose, that amount of toxin which, when mixed with one unit of standard antitoxin, is just sufficient to kill in four days a guinea pig approximately 250 grams in weight. With these limits established, the serum to be standardized is mixed with the toxin just titrated, and the largest amount of serum which, when mixed with the L+ dose of toxin, will produce a 100 per cent mortality in the inoculated guinea pigs is considered to contain one unit of diphtheria antitoxin.

A second method is based upon the observation that the intradermal injection of 1/250 to 1/500 MLD of diphtheria toxin into a guinea pig is followed by a local reaction, swelling and erythema and, with slightly larger amounts of toxin, necrosis, a phenomenon sometimes called the *Römer reaction.* By the use of such intradermal inoculations an Lr dose of toxin may be determined, *i.e.*, that amount of toxin which, when mixed with one standard unit of antitoxin, will produce the minimal skin reaction. The serum to be standardized is mixed in varying amounts with Lr doses of toxin, and that amount of serum which gives the minimal skin reaction is considered to contain one unit of antitoxin.[29] This method has the advantage of allowing the testing of a number of toxin-antitoxin mixtures in one animal but has not displaced the classic method in common usage.

The third method makes use of the *Ramon flocculation,* the *in vitro* precipita-

[28] *Cf.* Amer. Jour. Public Health, 1935, 25:712.
[29] Glenny and Allen: Jour. Path. and Bact., 1921, 24:61.

tion of toxin and antitoxin when mixed in optimal proportions.[30] Standard antitoxin is mixed with varying quantities of toxin, and the tube first, *i.e.*, in time, showing precipitation contains one Lf dose of toxin. Varying amounts of the serum to be standardized are mixed with the Lf dose of toxin. The amount of serum in the tube first showing flocculation in this second series is considered to contain one unit of antitoxin. This method differs from the other two in that it depends upon the combining power of a toxic filtrate rather than on toxicity. It is generally used, not as a final method of standardization, but as a preliminary to standardization by the Ehrlich method.

The interrelationships of these limits are of some interest. The L+ dose is, of course, larger than the L_0 dose, and, because of the peculiarities of the toxin-antitoxin reaction, by considerably more than one MLD. The Lr dose approximates the L_0 dose as might be expected in view of the small amount of toxin required to elicit the skin reaction. The Lf dose is generally somewhat less than any of these, since it is a measure of combining power rather than toxicity and is unaffected by differences in the proportions of toxin and toxoid. It would appear that the Lf/Lr ratio should be the same for a given toxic filtrate immediately upon standardization. It has been found, however, that this ratio differs with different antitoxic sera. This appears to be a consequence of differences in avidity, not of hypothetical toxin components as suggested by Ehrlich, but of antibody. The avidity of antitoxic sera is associated with the protein fraction containing the antibody; successive globulin fractions differ from one another in avidity (see also p. 314).

The Bactericidal Substances—Lysins. It was early shown by Nuttall[31] that fresh, defibrinated animal blood is markedly bactericidal. This property, also possessed by cell-free serum, was found to be heat-labile and destroyed by holding at a temperature of 55° to 56° C. for thirty minutes. Such heated sera are said to be *inactivated*. The blood or serum from a single animal species is not equally active on all species of bacteria and, conversely, a given bacterial species is affected to varying degrees by the blood of different animal species. It was suggested by Buchner that the natural resistance of an animal to infection could be explained in part by this bactericidal quality of the blood, and he proposed the name *alexin* (Gr., to ward off) for the heat-labile activity.

Although this bactericidal activity of the blood and serum of normal animals is frequently specifically increased by immunization, such an increase does not invariably accompany the development of the immune state. The serum of guinea pigs immunized with *Vibrio metchnikovii* is strongly germicidal for that microorganism, while that of unimmunized guinea pigs is devoid of specific bactericidal quality. On the other hand, immunization with streptococci, while producing a specific resistance to infection with the bacterium, frequently fails to induce an increased, specific germicidal activity.

Pfeiffer Phenomenon. The bactericidal effect is, in some instances, accompanied by visible dissolution of the bacterial cells. The course of events following the introduction of cholera vibrios into the peritoneal cavity of an immunized animal was followed microscopically by Pfeiffer[32] and described by

[30] Ramon: Compt. Rend. Soc. Biol., 1922, 86:661, 711, 813.
[31] Nuttall: Ztschr. f. Hyg., 1888, 4:353.
[32] Pfeiffer and Issaeff: Ztschr. f. Hyg., 1894, 17:355.

him in detail. The vibrios first lose their motility, then swell up and crumble into small fragments. The dissolution of the fragments follows, and no trace of the bacterial cell remains visible. This action of the body fluids is attributable to the presence of an antibody termed a *lysin* (Gr., to loose, dissolve). It occurs not only within the peritoneal cavity of an immunized animal, but also when the peritoneal fluid or blood serum is removed from the body and brought immediately in contact with the bacteria *in vitro*. The lytic activity of bactericidal sera *in vitro* is heat-labile and the process of lysis appears to be essentially identical with that observed *in vivo*.

Visible lysis of bacterial cells, however, does not invariably accompany the lethal activity of a bactericidal serum. In fact, the majority of bacteria are not dissolved by immune sera in the manner described above. This is not to be taken to indicate that the processes are fundamentally different; not only are many bacteria highly resistant to visible structural alterations, *viz.*, the lack of sensitivity to wide variations in osmotic pressure, mechanical pressure, etc., but, as will appear, the antigen-antibody reaction takes place in two stages, the first the union of antigen and antibody and the second the visible consequences of that union. Whether or not dissolution of the bacterial cells occurs, it may be readily shown that they unite with the lysins.

Hemolysis. The lysis of bacteria by an immune serum is not a unique reaction in which only bacterial cells may play a part; bacteriolysis is, rather, a special case of a general phenomenon, for immunization with a variety of cells results in the production of cytolytic sera. Of these, erythrocytes have been by far the most widely studied, for the lysis of these cells, hemolysis, is readily apparent in the test tube; the red opacity of the cell suspension changes to the clear red solution of hemoglobin as lysis proceeds. The stroma is not dissolved but, upon examination, appears misshapen. First demonstrated by Bordet,[33] hemolysis has been particularly useful not only as a type of lytic reaction peculiarly suitable for laboratory manipulation, but also as an indicator of antigen-antibody combination when visible lysis does not or cannot take place. It may be noted that the immune hemolysins, *i.e.*, those formed by the animal body in response to the injection of erythrocytes, are to be distinguished from the filterable hemolysins formed by bacteria (p. 206).

Antisera have been prepared against a variety of other cells. The injection of spermatozoa leads to the appearance, in the serum of the inoculated animal, of a spermatoxic substance that first renders the corresponding spermatozoa motionless and then kills them. A number of similar cytolytic sera have been prepared—"nephrotoxic" sera by the injection of kidney cells, "hepatotoxic" sera by the injection of liver cells, etc., but the organ specificity claimed for these last has not been satisfactorily demonstrated.

The Mechanism of the Lytic Reaction. An observation contributing in large measure to a partial understanding of the mechanism of the action of the immune lysin was that lytic or bactericidal activity could be completely restored to an inactivated serum by the addition of a small amount of fresh unheated serum, either normal or immune. It appears, therefore, that the

[33] Bordet: Ann. Inst. Pasteur, 1898, *12*:688.

lytic reaction is a consequence of the interaction of three components, rather than two as in the case of the toxin-antitoxin reaction. One of these is clearly an immune body, *i.e.*, is formed in response to the injection of the antigen, and another, the heat-labile component present in normal as well as immune serum. The third component is, of course, the antigen.

The heat-labile component was termed *alexin* by Buchner,[34] as indicated above, and renamed *complement* by Ehrlich. The immune body or antibody was called *substance sensibilisatrice* or *sensitizer* by Bordet and *amboceptor* by Ehrlich. The relation between these components may be expressed in tabular form for a hemolytic system

erythrocytes + unheated immune serum = hemolysis
(antigen)   (complement or alexin
            + amboceptor or sen-
                sitizer)
erythrocytes + heated immune serum = no hemolysis
(antigen)   (amboceptor or sensiti-
                zer)
erythrocytes + heated immune serum + unheated normal serum = hemolysis
(antigen)   (amboceptor or sen-   (complement or alexin)
                sitizer)

These components react with one another, not at random but in an orderly manner; the union between antigen and amboceptor must precede the reaction with complement. Complement does not combine with antigen in the absence of amboceptor, but antigen and amboceptor will unite regardless of the presence of complement. This may be demonstrated directly by using mixtures of only two components or in the presence of all three by holding the mixture at 0° C.; the antigen-amboceptor union readily takes place at this temperature, but complement unites only slowly and may be demonstrated in the supernatant following centrifugation.

According to Ehrlich, complement acts upon the antigen only indirectly through the amboceptor, the last functioning as a bridge between the first two. In this connection it is of interest that colloidal silicic acid may act as a senstitizing agent in the lysis of red cells. The evidence does not require that the action of complement be indirect in this way; it is established only that the antigen must be sensitized by the antibody before union with complement can occur. According to Bordet the union is in the nature of a specific adsorption, the sensitized antigen being rendered susceptible to the lytic action of the complement. For example, it is well known that tannic acid will act as a sensitizer, and treated erythrocytes are lysed in the presence of complement. The evidence, which cannot be considered at length here, strongly supports this view. In consequence it is generally admitted that the terms alexin and sensitizer are preferable to complement and amboceptor, although the latter remain in common usage. Quantitative studies by Heidelberger and his co-workers[35] have shown that under ordinary circumstances

[34] Buchner originally used the term alexin to designate the entire bactericidal action of the blood serum; alexin was later used by Bordet for the heat-labile substance alone; and at the present time alexin is regarded as synonymous with complement.

[35] Heidelberger: Jour. Exp. Med., 1941, 73:681; Heidelberger, Weil and Treffers; *ibid.*, 1941, 73:695; Heidelberger, Rocha e Silva and Mayer: *ibid.*, 1941, 74:359.

about seven molecules of complement are required for each molecule of antibody, but in very dilute solution the ratio may approach unity. These workers believe that complement exists in a loose union with antibody but is tightly bound in the antigen-antibody complex.

The Neisser-Wechsberg Phenomenon (Complement Deviation). It was observed by Neisser and Wechsberg that when varying amounts of immune serum (amboceptor) are added to constant amounts of normal serum (complement) and antigen, there is, as might be expected, no lysis with very small amounts of amboceptor, but as larger amounts are added, lysis takes place. When, however, amboceptor is added in considerable excess, lysis again fails to take place. From these and similar experiments it was concluded that a "deviation of complement" occurs under conditions where the amboceptor is in great excess, *i.e.,* the complement unites with the unbound amboceptor rather than with the amboceptor which has united with the antigen. A special explanation need not be devised for this phenomenon, however, for a similar failure of antigen-antibody union to occur is apparent in other serological reactions in the region of antigen or antibody excess. Such, for example, is the prozone phenomenon (p. 301) in the precipitin and agglutination tests. The same effect may be observed *in vivo,* for successful protection of mice against pneumococcus infection has long been known to be dependent upon optimal amounts of antiserum and culture; otherwise a zonal phenomenon in which there is no protection (the *Schwellenwert* of Neufeld and Haendel) may be encountered.

The Bordet-Gengou Phenomenon or Complement Fixation. As pointed out above, the lytic action of an immune serum is difficult to observe with many bacteria and cannot take place with an antigen such as egg albumin. Whether or not the phenomenon of lysis occurs or is made evident through some observable change in the antigen, it is possible to demonstrate the union of antigen and antibody by the addition of an indicator system. The lysis of red blood cells is used for this purpose and is commonly referred to in this connection as the hemolytic system; sheep cells and sheep hemolysin are generally used.

The antigen and inactivated serum (either of which may be unknown) are mixed together with the proper amount of complement in the form of fresh, unheated guinea pig serum. If the antigen and the inactivated serum unite, the complement will combine with the sensitized antigen and is said to be "fixed." When the hemolytic system is added in the form of heated hemolytic serum and erythrocytes, no complement is available, hemolysis does not occur, and the test is positive. If, however, the antigen and inactivated serum do not react, the complement remains free and combines with the sensitized red cells, hemolysis results and the test is negative. This phenomenon may be illustrated in the following way:

$$\begin{array}{ccc}
\text{antigen} \quad \text{positive} & \text{negative} & \text{sheep red cells} \\
+ \quad \longleftarrow \text{---complement---} \longrightarrow & + \\
\text{inactivated serum} & \text{inactivated hemolytic serum} \\
& \text{(amboceptor)}
\end{array}$$
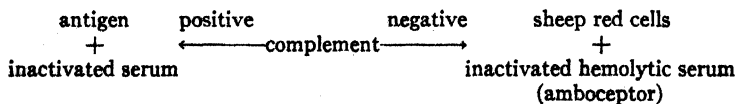
The quantitative aspects of the test are obviously of primary importance,

and each reagent must be titrated and added in the proper amount. As usually carried out, the degree of hemolysis is estimated as O, $+$, $++$, $+++$, or $++++$, the last representing complete hemolysis. A considerably more accurate end point is that of 50 per cent hemolysis, measured in a photo-electric colorimeter or spectrophotometer. A quantitative complement fixation which allows a close approximation of amount of antibody has been developed,[36] in which an excess of complement is added to the antigen-antibody mixture and the amount remaining after fixation titrated with the usual hemolytic system to a 50 per cent end point.

The complement-fixation test may be used with a known antiserum for the identification of an unknown bacterium or with a known antigen as a means to detect antibodies in an unknown serum. Perhaps the commonest application of the test is in the diagnosis of syphilis, where it is known as the Wassermann test.

The Nature of Complement.[37] It is likely that the process of lysis is accomplished by complement, the antibody simply sensitizing the antigen to this lytic action. Complement is not increased during immunization, and a higher titer immune serum may be only feebly lytic because there is not sufficient complement present to utilize the excess of amboceptor. It is of some interest that there is little species specificity in complement; that from guinea pig serum, for example, may bring about the lysis of beef corpuscles in the presence of amboceptor in goat serum. Some quantitative differences are apparent, however, for a serum which may be actively complementary in some reactions may be relatively inactive in other combinations.

The property of inactivation by heat has been referred to above. Complement is also inactivated by shaking, but in neither case is the inactivation completely irreversible, for some activity may be regained on standing. In this respect complement behaves as a typical hydrophilic colloid which can be made to aggregate by physical forces but shows a tendency to spontaneous dispersion and restoration to the original state. The activity disappears upon standing—rapidly at room temperature (two or three hours) and more slowly in the ice-box, where it may be preserved for three or four days. Complement is irreversibly destroyed by strong acids or alkalis and reversibly inactivated by ions such as Mg, Ca, Ba, Sr and SO_4 or by hypertonic salt solutions. In this connection it is of some interest that complement which is inactivated by raising the salt concentration to 5 to 10 per cent may be preserved at low temperatures in this form for several weeks, the activity being regained upon dilution with distilled water. These properties and some others, such as ready adsorption on surfaces, suggest a close relationship between complement and the enzymes; it has also been pointed out that there are remarkable resemblances to certain compounds of protein with soaps and lipids. An association of complement with the blood-clotting mechanism is suggested by the observations that many substances have both anticoagulant and anticomplementary activity, and that there is a close correlation between thromboplastic and complement-fixing power of tissue ex-

[36] Mayer, Osler, Bier and Heidelberger: Jour. Immunol., 1948, 59:195.
[37] See the review by Pillemer: Chem. Rev., 1943, 33:1.

tracts and sera. It seems clear, however, that complement is not identical with prothrombin, and more recent work[38] suggests that inactivation of complement of plasma blocks the conversion of prothrombin to thrombin. The nature of the lytic action of complement is as yet, however, purely speculative.

Complement is intimately associated with the serum proteins and there is reason to believe, *viz.* the destruction of complement by trypsin and the antigenicity of the activity, that this lytic agent is protein in nature. Complement may be split into two parts by the separation of serum protein into albumin and globulin. The globulin fraction will unite with sensitized cells although no lysis occurs, and is called the *mid-piece*, while the albumin fraction which will not unite with the sensitized antigen in the absence of the mid-piece but produces lysis when it unites with the sensitized cell-mid-piece complex is termed the *end-piece*. Other investigations have indicated that complement may be split up into four components by other fractionation methods. These are:

C'_1 or mid-piece which is precipitated from guinea pig serum by passing CO_2 through the serum diluted 1:10 with distilled water, or by dialysis against distilled water. This component is destroyed at 56° C in 30 minutes and is a euglobulin containing carbohydrate.

C'_2 or end-piece which remains in solution after the C'_1 fraction is precipitated. It shows the same heat lability and is a part of a mucoglobulin fraction containing 10 per cent carbohydrate.

C'_3 is inactivated by yeast, zymin or cobra venom and is not destroyed at 56° C in thirty minutes.

C'_4 or fourth component is specifically inactivated by treating guinea pig serum with dilute ammonia, hydrazine or viper venom, or by shaking with chloroform or ether. It is also heat-stable but this stability is greatly reduced in the presence of 10 per cent sodium chloride. It appears to be a carbohydrate portion of the mucoglobulin fraction containing C'_2 whose carbonyl groups are attacked by ammonia.

Human complement likewise consists of these four components and they are mutually interchangeable with those in guinea pig serum.[39] The four components are also present in frog serum and carp serum and are either identical or closely related to those of guinea pig serum.[40] The titer of complement is, of course, limited by the fraction occurring in smallest amount; this appears to be C'_2 in human serum, and C'_3 in guinea pig serum. All four components are necessary for lysis of red cells and bactericidal action, but all are not equally fixed by the antigen-antibody complex. In general, relatively large amounts of C'_1 and very small amounts of C'_3 are fixed, but the relative fixation depends upon what other components are present.

Opsonins. If a mixture of polymorphonuclear leucocytes and bacteria or other particulate matter is incubated for a time, it will be found on microscopic examination that a number of the leucocytes have ingested the foreign particles. Few if any particles will be ingested if the mixture is prepared in physiological salt solution, a considerable number if the fluid is normal serum. In

[38] Mann and Hurn: Proc. Soc. Exp. Biol. Med., 1948, 67:83.
[39] Ecker and Seifter: Proc. Soc. Exp. Biol. Med., 1945, 58:359.
[40] Cushing: Jour. Immunol., 1945, 50:61, 75.

the case of bacteria, great numbers of the microorganisms will be found packed into the leucocytes when the two are suspended in the specific immune serum. The antibodies present in the immune serum which so remarkably stimulate this engulfment by body cells are designated *bacteriotropins,* a term not in common use, or *opsonins.* The term opsonin was originally used to designate the activity of normal serum, hence the antibodies in the immune animal are sometimes called immune opsonins. The cells which ingest such particulate matter are termed *phagocytes* (devouring cells). This property is not confined to the polymorphonuclear leucocytes or heterophils, although these have been most widely used in *in vitro* experiments because of their availability, but is
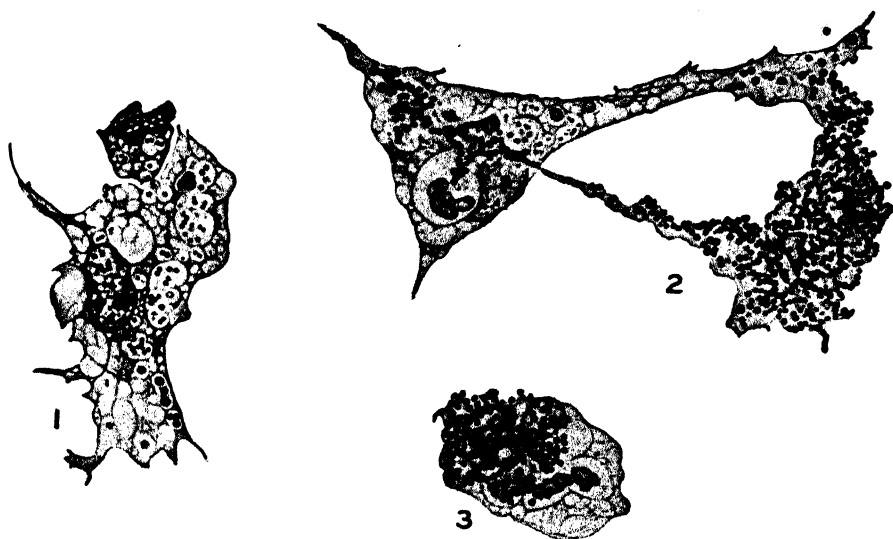
Fig. 34. Phagocytosis of pneumococci by culture macrophages. *1,* Phagocytosis in presence of normal serum; relatively few bacteria have been ingested. 2 and 3, Phagocytosis in the presence of antipneumococcus serum; enormous numbers of pneumococci have been ingested and appear as agglutinated masses. The lightly staining forms are degenerating. Hematoxylin and eosin-azure II. × 1200 (Zuckerman).

also present in various mononuclear phagocytes. These cells and their role in immunity are discussed in the following chapter.

The Opsonic Index. A quantitative estimate of opsonin present in a given immune serum may be made by comparing the number of bacteria ingested by normal leucocytes in normal serum with the number ingested by normal leucocytes in the immune serum. The appropriate mixtures are prepared and incubated in capillary tubes, smears made and stained, and the bacteria engulfed by an arbitrary number (usually 50 or 100) of leucocytes counted.[41] The average number of bacteria per leucocyte, or *phagocytic index,* is determined for the normal and immune sera, and the ratio of the phagocytic index of the immune serum to that of the normal serum is termed the *opsonic index.*

[41] The details of this technique may be found in Wright and Colebrook: *Technique of the Teat and Capillary Glass Tube.* 2nd ed. Constable and Co., Ltd., London. 1921; Fleming: *A System of Bacteriology.* Medical Research Council, London. 1931, 9:212.

The opsonins may be estimated also by the dilution method; specimens are prepared in the usual way, except .that the normal and patient's sera are diluted with saline or Ringer's solution. One mixture is made with salt solution or Ringer's solution to determine the degree of spontaneous phagocytosis. The dilution of serum which gives the same amount of phagocytosis as a mixture without serum is taken as the end point.

The Factors Influencing Phagocytosis. The process of phagocytosis is markedly influenced by environmental factors and the nature of the bacterium and leucocytes used, as well as by the amount of opsonin present. Departures from a neutral or very slightly acid reaction or from isotonicity, and the presence of certain ions, notably the citrate radicle, depress the degree of phagocytosis. The last is of practical significance in that it contraindicates the use of citrated blood. The presence of calcium, on the other hand, may restore phagocytic power to leucocytes which have been allowed to stand in isotonic salt solution for a number of hours, a point of some interest in connection with the apparent intimate relation between this ion and cell division and the ability of ameboid cells to form new surfaces.

The nature of the bacterium to be ingested is of considerable importance; in general, virulent forms are relatively resistant to phagocytosis. It is not unlikely that this resistance is associated with the presence of a capsule; in the case of the pneumococcus, for example, not only are the smooth, encapsulated forms highly resistant to phagocytosis, but the presence of capsular polysaccharide markedly inhibits phagocytosis by an immune serum, presumably through combination with the antibody. Bacteriophage (p. 916) renders bacteria considerably more sensitive to phagocytosis.

The phagocytic power or activity of the leucocytes is subject to considerable variation independent of variation in the opsonic content of the blood. This inherent phagocytic power of the leucocytes varies, with respect to certain bacteria at least, even in persons apparently in perfect health. In the child at birth the leucocytes are somewhat less active phagocytically than in the adult; they grow less active for a few months, and then more active, the adult standard for streptococci, pneumococci and staphylococci being reached about the third year. In pneumonia, scarlet fever and other conditions in which there is acute leucocytosis when the outlook is favorable, the phagocytic power of leucocytes has been found to be greater than normal for the specific bacteria. The increase in activity in such cases may be due to the predominance of young leucocytes.

The Process of Phagocytosis. The mechanism of ingestion is essentially one of the interplay of interfacial forces. A formulation of the free surface energy relationships at the points of contact between particle and cell and their respective liquid interfaces has been worked out by Fenn[42] and subjected to experimental tests by Mudd[43] and his associates. Low interfacial tension of the bacteria against the leucocytes—and high interfacial tension against the medium—favors ingestion. The immune opsonins, and to some extent the

---

[42] Fenn: *The Newer Knowledge of Bacteriology and Immunology.* Jordan and Falk, University of Chicago Press, Chicago. 1928. p. 861.

[43] The process of phagocytosis is discussed at length by Mudd, McCutcheon and Lucke: Physiol. Rev., 1934, 14:210.

"normal" opsonins, apparently form a surface deposit on the bacterial cells which promotes phagocytosis by altering the interfacial tension in this manner. In addition to surface tension, other factors such as the viscosity of the phagocytic cell substance enter into this phenomenon. Physical tensions are not the sole controlling factors, however, for increased oxygen consumption accompanies the process of ingestion, the rise beginning at once, reaching a maximum value twice that at the start in about fifteen minutes and persisting for 90 to 150 minutes.

It is of some interest that bacterial cells may be artificially "opsonized," *i.e.*, made more readily phagocytable, by treatment with iron ammonium alum, chrome alum, protamine sulfate or gallotannic acid. The effect is reversed by treatment with oxalate but reversion is very difficult when the cells are sensitized with immune opsonin. The significance of such observations in immune opsonization and phagocytosis is not clear.

The work of Wood and his co-workers[44] has shown that phagocytosis may occur quite as readily in the absence of antibody as in its presence, provided that it occurs on a suitable surface; most body tissues provide such surfaces, *viz.*, the phagocytosis of pneumococci on the alveolar surfaces of the lungs. This phenomenon of "surface phagocytosis" has not been previously described.

The Fate of Ingested Bacteria. Following phagocytosis many, but not all, species of bacteria may be observed to undergo a process of dissolution, with swelling, granulation and fragmentation appearing as successive stages in their destruction. Although it was early supposed that intracellular digestion was no more than intracellular lysis through the agency of the immune lysin and complement which would have occurred whether or not phagocytosis took place, it now seems probable that the two processes are essentially different.

Bacteria are no exception to the rule that living organisms are not subject to attack by digestive enzymes, and the question arises as to whether death is a necessary preliminary to ingestion or whether it may occur within the phagocyte. It is probable that the viability of the microorganism is not an important factor in phagocytosis; in most cases the cell may be engulfed whether it is living or dead. In the case of those bacteria which are destroyed within the leucocyte, killing is necessary before digestion can take place. Killing of a viable organism is accomplished through the agency of endocellular bactericidal substances which have been designated *leukins*. These substances are not equally active on all species of bacteria, some microorganisms remaining viable within the phagocyte over considerable periods of time. The ingested bacteria are protected against the action of immune serum, *i.e.*, bactericidal or lytic antibodies, and this is possibly of some significance in the dissemination of bacteria within the host in the event that they remain viable and are later freed from the leucocyte. Leucocytes have been shown to contain a variety of digestive enzymes, including proteases, lipases and various carbohydrate-splitting ferments which are presumably responsible for the actual dissolution of the bacterial cell.

The Nature of the Opsonic Activity. The opsonins are true antibodies in that they are increased by immunization and exhibit the specificity characteristic of the immune antibodies. The activity is, like that of the lytic sera, made

[44] Wood, Smith and Watson: Science, 1946, 103:28.

up of two components, one thermostable and the other thermolabile. The thermolabile component is present in normal serum and the reduced opsonic powers of an inactivated immune serum may be restored by the addition of a small amount of normal serum. In this and other respects the thermolabile component of opsonin strikingly resembles complement and in the past has been assumed by many to be identical with this component of the lytic system. In general, however, it has not been possible to show any constant relation between the components of complement and opsonization. For example, the fourth component of complement has been reported[45] to be necessary to the lytic reaction but not required for opsonization, but Ecker[46] has reported that the thermolabile opsonin of the human serum is identical with C'_1, C'_2 and C'_4 in combination but not separately or in any combination of two. It ap-
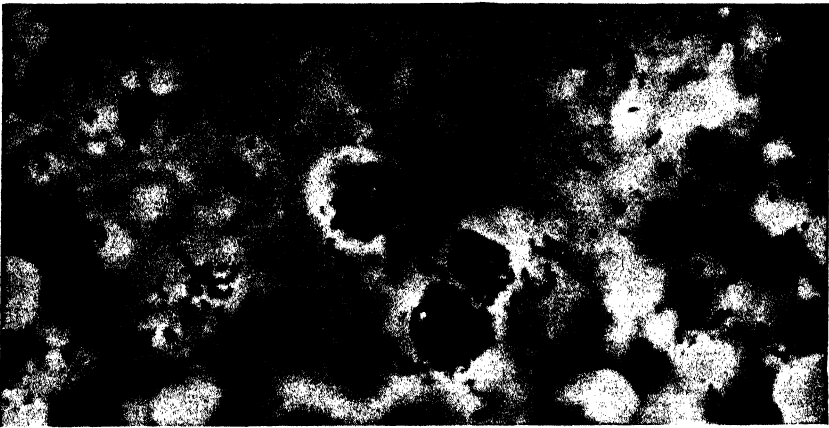
Fig. 35. The phagocytosis of typhoid bacilli by leucocytes in whole blood. Note the enormous numbers ingested by the white cells and the bacilli lying free. Hastings' stain; × 1200.

pears, then, that complement and the thermolabile element of opsonin, while resembling each other closely in many respects, may not be regarded as identical.[47]

There is some question as to whether the thermolabile factor is essential to phagocytosis, for experimental evidence has been presented[48] which indicates that while the thermolabile factor contained in normal serum markedly accelerates the *rate* of phagocytosis, approximately the same number of bacteria are ingested at the end of eight hours by phagocytes in the presence of heated serum alone.

Agglutinins. If the blood or serum of an animal previously immunized against a bacterium be mixed with a suspension of the microorganisms, the latter become immobilized and in a short time aggregate to form large clumps of cells. In the test tube these clumps settle out, and the turbid bacterial sus-

[45] Gordon, Whitehead and Wormall: Jour. Path. and Bact., 1929, 32:57.
[46] Ecker and Lopez-Castro: Jour. Immunol., 1947, 55:169.
[47] See also Ecker, Pillemer and Kuehn: Jour. Immunol., 1942, 43:245.
[48] Ward and Enders: Jour. Exp. Med., 1933, 57:527.

pension is cleared with the formation of a precipitate-like mass of clumped cells in the bottom of the tube. This phenomenon is termed *agglutination* and the bacterial cells are said to be *agglutinated*. The bacteria are not killed by agglutination and will, in fact, grow in immune serum although with altered morphology and the formation of long chains of bacillary forms—the so-called "thread reaction" of Pfaundler. Living bacteria need not be used for the agglutination reaction, for dead bacteria are agglutinated as readily as the viable forms.

Although many species of bacteria may be clumped by "normal" sera in low (1:5 to 1:10) dilutions, the capacity of a serum to agglutinate bacteria is greatly enhanced by immunization; high-titered antisera may be prepared which will bring about agglutination in dilutions of 1:20,000 to 1:50,000. The agglutination reaction is, then, an antigen-antibody reaction and the antibody is designated an *agglutinin*. The antigen is sometimes termed an *agglutinogen*. The agglutinin does not require the cooperation of complement or other heat-labile substances and inactivated sera will agglutinate to titer.

Not only bacteria but a variety of free cells, including erythrocytes and others, are agglutinated by normal and immune sera. The incompatibility of human blood groups is a consequence of the presence of hemagglutinins. Hemagglutinins are also formed by some bacteria and may possibly be instrumental in the formation of the thrombi observed in the blood vessels after death from certain of the infectious diseases.

Like the other immunological reactions, agglutination is highly specific, an antiserum agglutinating only the homologous antigen. Unlike the lysins and opsonins, however, the activity is heat-stable and the antibody does not require the cooperation of a heat-labile component. The reaction may be observed microscopically by mixing a suspension of bacteria and diluted antiserum on a slide but is most commonly carried out by mixing the two in 0.5 to 1.0 ml. amounts in small test tubes and observing the formation of a precipitate. In the latter instance, varying dilutions of serum (frequently prepared in geometrical progression by mixing with an equal amount of physiological salt solution, *i.e.*, 1:10, 1:20, 1:40, 1:80, etc.) are added to the bacterial suspension and incubated at 37° overnight or at 55° C. for two hours. The highest dilution showing observable flocculation is taken as the titer of the serum; a serum showing agglutination in a dilution of 1:10,000 but not in 1:20,000 is said to have an agglutinin titer of 1:10,000. Such titers are variable to some degree depending upon the density of the bacterial suspension and other factors; light suspensions, for example, showing only a faint turbidity to the eye will give higher agglutinin titers than heavy suspensions.

Cross Reactions. Although the agglutination reaction is highly specific, certain cross reactions between closely related bacterial species are frequently observed. Such reactions are attributable not to a lack of immunological specificity but to the immunological heterogeneity of the bacterial cell. As indicated above, the cell is made up of a variety of antigenic components, an "antigenic mosaic." Clearly, then, if the same component is present in each of two species of bacteria, an antiserum prepared against the one will agglutinate the other but generally to a reduced titer, *i.e.*, only in the lower serum dilutions. This sharing of antigenic components and the resulting cross reactions

are particularly common among the paratyphoid Salmonella and dysentery bacilli. The agglutinins responsible for such cross reactions are sometimes referred to as "group agglutinins" and the phenomenon as "group agglutination."

Agglutinin Absorption. The direct demonstration of the antigenic heterogeneity of a bacterium and the consequent multiple antibody content of its homologous antiserum are made possible by agglutinin absorption. If a heavy suspension of bacteria is prepared in diluted (commonly 1:50) antiserum, incubated for two to three hours, and centrifuged, the supernatant diluted serum will be found to have lost its ability to agglutinate the bacterium with which it was absorbed; the agglutinins for that microorganism have been taken up by the bacterial cells, leaving other agglutinins intact. In practice it is necessary to absorb two or three times and, since the phenomenon is an adsorption, it is not always possible to remove completely the agglutinins in question.
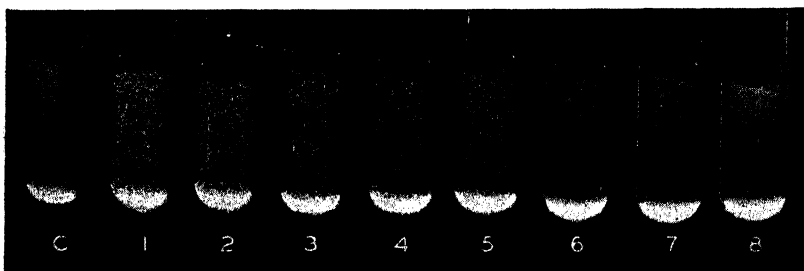
Fig. 36. The macroscopic agglutination test—flagellar agglutination of Salmonella. The control tube, C, contains bacterial suspension but no antiserum. Successive dilutions in the numbered tubes are 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10,000, and 1:20,000. Note agglutination in 1:10,000 but not in 1:20,000.

This preferential absorption makes possible the breakdown of the antigenic mosaic of the bacterial cell into component parts. For example, an antiserum prepared against a bacterium containing antigens *A, B* and *C* will contain antibodies *a, b* and *c.* If such an antiserum is absorbed by a second bacterium which contains antigens *B* and *C,* antibodies *b* and *c* will be removed leaving *a* intact, and the antiserum will still agglutinate its homologous bacterium. Suppose an antiserum is prepared against the second bacterium which will contain antibodies *b* and *c.* If this serum is absorbed by the bacterium containing antigens *A, B* and *C,* it will no longer agglutinate its homologous antigen, for all the antibodies will have been removed. Clearly, then, the complete absorption of a known serum by an unknown organism does not indicate that the unknown organism is necessarily immunologically identical with that with which the known serum was prepared; to prove such immunological identity a "mirror absorption" must be carried out, *i.e.,* each antiserum absorbed with each organism.

The agglutination test and agglutinin absorption, although used to some extent in the diagnosis of infectious disease (as in the Widal test in which typhoid bacilli are mixed with patient's serum), have been found particularly valuable in the study of the relation of bacterial species to one another and in

some instances, as in the case of the Salmonella (Chapter 19), complex anti-
genic formulae have been worked out. The determination of the components
of the antigenic mosaic of a bacterial species is termed *antigenic analysis*.

The Prozone Phenomenon. It is not infrequently observed that immune sera
showing high agglutinin titers fail to agglutinate the homologous bacteria
in low dilutions, *i.e.*, 1 : 100 or less. This portion of the dilution range is
designated as the *prozone* or proagglutinoid zone. Prozones, although occa-
sionally observed with fresh sera, are more common and extend over a wider
range with old or heated sera. The prozone observed with fresh serum is com-
monly assumed to be associated with the presence of complement and some
other factor, while that shown by heated serum is attributed to the combina-
tion of serum albumin with immune globulin or with the antigen-antibody
complex in such a way as to prevent aggregation.[49]

"H" and "O" Agglutination. A number of bacterial species, particularly
Salmonella, Proteus and certain others, may agglutinate in one of two ways.
Macroscopically the "H" agglutination gives rise to a loose, flocculent pre-
cipitate, and upon microscopic examination it may be observed that the bac-
terial clumps are loose, the flagella of the microorganisms being entangled
with one another. The "O" agglutination, on the other hand, produces a finely
granular precipitate in which the individual bacterial cells are closely packed
together. These types of agglutination have been shown in micromotion pic-
tures by Pijper.[50] They are a consequence of the presence or absence of the
flagellar, heat-labile "H" antigen. Immunization with the whole bacterial cell
containing both flagellar and somatic antigenic components gives rise to anti-
sera containing both types of antibody, the "H" generally in high titer while
the "O" antibody is commonly active in dilutions of less than 1 : 1000.

Spontaneous Agglutination. Some strains of bacteria do not form stable
suspensions and are said to be spontaneously agglutinable. This behavior is
particularly characteristic of rough variants and, although not an immunological
phenomenon, is frequently of practical importance in agglutination studies.

Cold Agglutinins. Autohemagglutinins which clump red cells at 0° C. but
not at 37° C. have been described as occurring in primary atypical pneumonia
(p. 870) and a variety of other conditions including trypanosomiasis, mumps,
hemolytic anemia. The significance of these agglutinins and their relation to
the usual immune agglutinins is not clear[51]; possibly the phenomenon is re-
lated to the antibody-like activity of so-called acute phase serum (p. 306).

The Mechanism of Agglutination.[52] The clumping of bacteria under the
influence of immune serum may be taken as evidence *per se* that a force attract-
ing the cells to one another is operative at least at times. Similarly, the fact
that bacteria are not in a constant state of agglutination is indicative of a force
which tends to hold the cells apart from one another. The attractive or cohesive
force is probably that of surface tension, *i.e.*, the interfacial tension at the cell
surface, and the repulsion that of like electrical charges, for, as pointed out
previously, bacteria are negatively charged at *p*H's compatible with viability.

[49] See the discussion by Hayes: Brit. Jour. Exp. Path., 1947, 28:98.
[50] Pijper: Jour. Bact., 1941, 42:395.
[51] For instance, see Finland, Peterson and Barnes: Jour. Clin. Investig., 1945, 24:474.
[52] For the literature to 1919 see Buchanan: Jour. Bact., 1919, 4:73.

On the basis of such reasoning it would appear that the balance between these opposing forces determines whether the microorganisms will form a stable suspension or whether they will clump together and settle out.

It was early observed that the presence of an electrolyte is essential to agglutination; if both immune serum and bacterial suspension are dialyzed free of salt before mixing, the cells are not agglutinated, but if a trace of salt is added to the mixture, agglutination takes place at once. This behavior, it will be seen, corresponds to that of a mixture of two colloids of opposite charge,
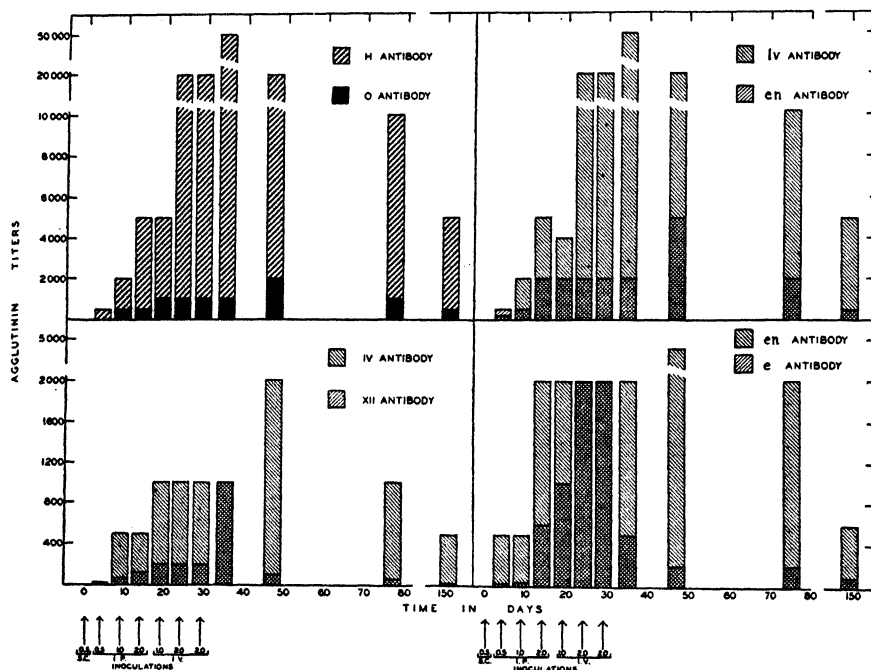
Fig. 37. The immune response in the rabbit to the antigenic complex of *Salmonella brandenburg*. Upper left, H and O agglutinin titers, showing the usual marked discrepancy between the two. Lower left, the differential response to the two components of the O antigen. Upper and lower right, the response to the components of the H antigen. Note the independent behavior of the antibodies as indicated by variation in peak titers. All titers determined by direct agglutination of heterologous antigens without agglutinin absorption.

such as gum mastic and gelatin, when the one is added in too small an amount to precipitate in the absence of salt. That the repelling effect of electrical charge is an important factor is also indicated by the agglutination of bacteria in the absence of antibody when the pH is lowered to the isoelectric point of the cells, a phenomenon termed *acid agglutination*. It was formerly thought that bacterial species might be sharply differentiated from one another on the basis of the pH of their acid agglutination, but this has not proved to be true.[58]

[58] For a summary of the literature see Gouwens: Jour. Inf. Dis., 1923, 33:113.

The agglutination of bacterial cells is, however, a function not only of electrical charge on the cells but also of the cohesive forces tending to draw them together. The studies of Northrup and DeKruif[54] in which both potential difference (between the cells and the suspending medium) and cohesive force were directly measured have shed considerable light on the mechanism of agglutination. These workers found that electrolytes in low concentration (0.01 N) affect primarily the potential, and in high concentration also decrease the cohesive force. If the cohesive force is not affected, agglutination occurs when the potential is reduced below the critical point of 15 millivolts. If, however, the cohesive force is decreased, the critical potential is also decreased and, therefore, in concentrated salt solutions agglutination does not take place even though there is no measurable potential. Immune serum, presumably adsorbed on the surface of the bacterial cells, while reducing the charge somewhat, appears to function by preventing the salt from decreasing the cohesive force, and agglutination occurs at the critical point of 15 millivolts. Furthermore, if the potential difference is reduced by electrolyte to 15 millivolts or less in a bacterial suspension, the addition of immune serum raises the reduced cohesive force and agglutination takes place. Examination of typhoid bacilli by the electron microscope in the presence of immune serum has shown that the flagella become thickened by the deposition of an antibody film approximately 21 Å thick and the cell walls become more opaque and less definite in outline. The serum-sensitized surfaces appear to be sticky, not only for one another, but also for other particulate matter.[55]

The presence of antigenic cell components may interfere with or prevent agglutination of bacteria by homologous antiserum. The presence of Vi antigen (p. 451) in typhoid bacilli, for example, renders them inagglutinable with homologous O antiserum, but they become agglutinable as the interfering antigen disappears during successive subcultures. Similarly, colon bacilli contain a thermolabile component of the envelope antigen (p. 424), the L antigen, which inhibits agglutination with O antiserum, but the bacterial suspension becomes agglutinable when this component is destroyed by boiling. Possibly the blocking effect is physical in nature in that the O antigen is covered, though it would seem that the forces operative in antigen-antibody union would be effective over the distance imposed by a monomolecular layer of immune globulin.

Precipitins. The mixture of an immune serum prepared against a soluble antigen, such as egg albumin, with its homologous antigen results in the formaation of a precipitate. This phenomenon is termed the *precipitin reaction* and the antibody a *precipitin;* the antigen is sometimes designated as a *precipitinogen.* The physical state of the antigen used for immunization is not of particular importance; antibacterial sera, for example, will give precipitates when mixed with preparations of the soluble cell substance of the microorganisms, and precipitins may almost always be demonstrated in lytic, antitoxic, opsonic and agglutinating antisera. It has been found in the first instance that the precipitate will fix complement and the flocculation of toxin-antitoxin mixtures has been referred to above. Like agglutination, precipitation does not occur in

[54] Northrup and DeKruif: Jour. Gen. Physiol., 1922, 4:639, 655.
[55] Mudd and Anderson: Jour. Immunol., 1941, 42:251.

the absence of electrolyte and does not require the presence of heat-labile sub-
stances such as complement.

In contrast to the agglutination test, the precipitin test is carried out with
undiluted, or only slightly diluted, serum but the antigen solution is diluted
in series. The reagents may be mixed in the usual way, or the antiserum
may be pipetted into very small test tubes and the solutions of diluted antigen
carefully layered on top with no mixing. In the first instance a precipitate is
formed which settles out like agglutinated bacteria, while in the second a pre-
cipitate forms at the interface between serum and antigen solution. The latter
test is termed a "ring test." The end point of either test may be taken as the
highest antigen dilution with which the serum forms an observable precipitate
and the titer of the serum given in these terms. Potent antisera may have ex-
tremely high titers, precipitating antigen in dilutions of 1:100,000 to 1:5,-
000,000. A more accurate measure of the precipitin content of an antiserum,
however, is that of the antigen dilution in which precipitation first occurs (and
generally is heaviest), for it is at this point, sometimes termed the *equivalence
zone*, that the antigen-antibody ratio is optimal for precipitation. Two such
optimal ratios may be distinguished, one when the antigen is held constant
and the antibody added in increasing amounts and the other when the anti-
body is constant and antigen is added. The two are not necessarily identical,
as might be expected, although very nearly so in the Ramon flocculation, in
which the end point is clearly that of optimum antigen-antibody ratio. In other
instances the amount of antibody required for most rapid flocculation when the
antigen is kept constant may be six to eight times as much as when the anti-
body is kept constant. The explanation of this phenomenon lies in the variable
proportions of antigen and antibody which make up the precipitate, as will
appear in a later section.

The precipitate formed consists of antigen and antibody in variable pro-
portions depending upon the relative amounts of the reacting substances. The
antigen is diluted rather than the antiserum because relatively large amounts
of antibody are required. As in the agglutination reaction, antibody is adsorbed
on to the surface of the antigen, but, since the antigen molecules are much
smaller than the bacterial cells, the surface to be covered is proportionally
greater and consequently large amounts of antibody are required. It follows,
of course, that the proportion of antibody to antigen is much greater in the
precipitate formed than in agglutinated bacteria.[56] The mechanism of the
precipitin reaction is probably essentially that of agglutination, molecules in-
stead of bacteria being clumped. It will be discussed at greater length in a
later section.

The close relation between the precipitation and agglutination reactions is
indicated by the agglutination by a precipitating antiserum of particles of inert
material, such as collodion, upon which the antigen has been adsorbed. These
particles then behave as particles of antigen and may be set up with serial dilu-
tions of antiserum as in the agglutination test.[57] Goodner[58] has carried out the
reaction in the reverse by adding collodion particles to the antigen-antibody

[56] *Cf.* Zinsser: Jour. Immunol., 1930, *18*:483.
[57] Cannon and Marshall: Jour. Immunol., 1940, *38*:365.
[58] Goodner: Science, 1941, *94*:241.

mixture, the complex being adsorbed and the particles agglutinated; he has called this "collodion fixation."

The precipitin reaction occurs not only between antibody and complete antigen but also with some partial antigens or haptenes. An antipneumococcus serum, for example, will react not only with a solution of the pneumococcus cell substance but also with pure capsular polysaccharide. When the haptene is of high molecular weight, as in the case of the polysaccharides, a visible precipitate is formed, but when it is a simple compound such as an organic acid or monosaccharide no observable precipitate appears. The reaction between antibody and such haptenes may, however, be shown by the fact that antisera treated with haptene will no longer precipitate the complete antigen; the antibody has already reacted with the partial antigen. The development of knowledge of the haptenes has made possible quantitative studies on the antigen-antibody reactions; when both reacting substances are proteins the two cannot be distinguished by chemical methods in a precipitate, but if the one be a polysaccharide and the other a protein, the reacting substances are readily differentiated and determined by ordinary analytical methods.

The precipitin reaction, like the other immunological reactions, is highly specific, but cross reactions are observed between chemically similar antigens that are analogous to "group agglutination." Such cross reactions occur between antisera for the blood proteins of man, chimpanzees, gorillas and various species of monkeys. An antihuman serum prepared by immunizing a monkey, however, does not show cross reaction with that monkey's serum. Other immunological relationships, such as those between the caseins of various animal species, the egg albumins, etc., appear in the precipitin reaction also. In most instances, however, the differentiation is sharp, or may be made so by the use of slightly diluted sera, and the simplicity and accuracy of the precipitin test have resulted in its frequent use in forensic medicine. The differentiation between chicken blood and human blood by the precipitin test in a case of suspected murder, for example, has the highest standing as evidence in court.

The studies of Landsteiner and others on the chemical basis of the specificity of antigens, which have already been discussed, have made use of the precipitin reaction almost entirely, and the similarities of molecular structure of antigens or the immunologically determinant groups provide a sound basis for the understanding of cross reactions.

Ablastin. The existence of an antibody which inhibits the reproduction of an invading microorganism was postulated by Ascoli[59] on the basis of his experiments with the anthrax bacillus. Similar results were reported later by Dochez and Avery[60] for the pneumococcus which suggested that immune serum temporarily inhibited the multiplication of these organisms and depressed their proteolytic and glycolytic activities. This effect was attributed to "antiblastic" or reproduction-inhibiting antibodies present in the immune serum. In reported experiments with bacteria, however, the results may be interpreted in other ways, and the existence of an ablastin for bacteria has not been conclusively demonstrated.

[59] Ascoli: Ztschr. f. physiol. Chem., 1906, 48:220; Centralbl. f. Bakt., Abt. II, 1908, 46:178.
[60] Dochez and Avery: Jour. Exp. Med., 1916, 23:61.

A reproduction-inhibiting antibody developed in response to invasion with certain of the parasitic protozoa, trypanosomes, was independently discovered by Taliaferro.[61] In this case the activity of the antibody was sharply differentiated from that of coexisting lysin and was demonstrable directly by cessation of cell division and indirectly in terms of variability in size of the microorganisms, *i.e.*, in a population of rapidly reproducing organisms the size of the individual cells is subject to wide variation whereas size is relatively constant in a population consisting entirely of adult forms. It is not necessary, however, to postulate the existence of an antibody which specifically inhibits reproduction, for the consequences of the union of antibody with the antigen of the living microorganism may well be such as to inhibit normal physiological functions including those associated with growth and cell division. For example, Nippostrongylus larvae in and about which immune precipitates have been formed are stunted and immobilized.

Perhaps related to ablastins are the "non-absorbable" antibodies which have been demonstrated in certain worm infections. Campbell[62] has found that antibodies to the larval tapeworm, *Cysticerus crassicollis*, present during the later stages of infection, cannot be absorbed from the serum by treatment with the parasites. He regarded these nonabsorbable protective substances as antienzymes, whose presence interferes with the processes of growth and reproduction, but which are not absorbed because the antigen is elaborated only by actively metabolizing cells and is not present in those which are physiologically quiescent.

Possibly to be regarded in the same light is the precipitin present in convalescent yellow fever serum which reacts with yellow fever serum taken during the acute stage of the disease but not with the virus.[63] This phenomenon, however, has been interpreted by some as indicative of the presence of a "pathological protein" in yellow fever. Avery and his co-workers[64] have observed a similar protein which appeared in the blood of human beings and of monkeys in various etiologically unrelated infections, and which formed a precipitate with the C substance (somatic polysaccharide) of pneumococci. Serum showing this activity has been called "acute phase serum." The C-reactive protein has been separated from serum proteins in crystalline form by McCarty[65] and shown to be immunologically specific.

Neutralizing Antibodies. In the light of the capacity of an immune serum to lyse, kill or sensitize bacteria to phagocytosis, it follows that the injection of a suspension of pathogenic bacteria in homologous immune serum may have less serious consequences to the susceptible animal than the inoculation of the bacteria alone. Since the usual serological reactions may be carried out with the filterable viruses only at considerable inconvenience owing to difficulties in the preparation of the antigen and the like (p. 853), it has become customary to evaluate the antibody content of antiviral sera in terms of

[61] *Cf.* Taliaferro: Amer. Jour. Hyg., 1932, *16*:32.

[62] Campbell: Jour. Immunol., 1938, *35*:195, 205, 465; Jour. Inf. Dis., 1939, *65*:12.

[63] Hughes: Jour. Immunol., 1933, *25*:275.

[64] Avery, Abernathy and MacLeod: Jour. Exp. Med., 1941, *73*:173 *et seq.* See also Löfström: Brit. Jour. Exp. Path., 1944, *25*:21; Löfström: Acta Med. Scand., 1943, Suppl., *141*:1; Hedlune: *ibid.*, 1947, Suppl. *196*:596.

[65] McCarty: Jour. Exp. Med., 1947, *85*:491.

their neutralizing capacities, *i.e.,* ability to prevent infection when injected with the infectious material. This technique is, however, not confined to the filterable viruses, for the "protective titers" of certain antibacterial sera, as, for example, in studies on the efficacy of typhoid immunization (p. 461), have been found a more satisfactory measure of antibody content than the measurement of agglutinin, precipitin or other single antibody titers. It is becoming more and more generally used as a method of assay of the potency of antisera.

In practice, varying amounts of immune serum are mixed with a constant amount of bacterial suspension or infectious material and injected into susceptible animals either with or without a period of preliminary incubation. In this way a crude approximation of the neutralizing or protective capacity of an immune serum can be made.

Whether the "virus neutralizing" or "protective" capacity of an immune serum can be accounted for on the basis of the combined activity of the other known antibodies or whether other factors are involved is open to question. Since, however, the differentiation of the kinds of antibodies from one another is largely a matter of the technique by which the antigen-antibody reaction is demonstrated, as will appear, the neutralizing or protective action of an immune serum which is not identical with precipitin, agglutinin or other antibody is legitimately regarded as a separate antibody.

The Nature of Antibodies. The question of the nature of the antibodies is one of no small practical as well as theoretical importance. These substances are intimately associated, if not identical, with the globulin fraction of the serum proteins and may be separated from the other serum constituents, sometimes by simple dilution with distilled water but more commonly by some salting out procedure. With one or two exceptions, the range of salt concentration over which they are precipitated is broad and not sharply defined. Attempts to further differentiate the active fraction into euglobulin and pseudoglobulin have not been successful, the antibody appearing sometimes in the one, sometimes in the other and not infrequently in both, depending upon the animal immunized. Electrophoretic fraction of serum globulin in the Tiselius apparatus, however, allows the separation of three components on the basis of mobility. These are designated α-globulin, β-globulin and γ-globulin in order of decreasing mobility. Antibody appears to be associated almost exclusively with the γ-globulin fraction, though β-globulin may occasionally show some activity. Probably the purest antibody preparations have been those obtained by dissociating antigen-antibody precipitates, the antibody having been previously purified by salting out; about 0.01 mg. of globulin per unit of diphtheria antitoxin is precipitated by toxin. Northrup[66] has prepared crystalline diphtheria antitoxin which appears to be a pure protein, crystallizing in thin plates and containing 700,000 to 1,000,000 antitoxic units per gram. It has not been possible as yet, however, to separate antibody from serum globulin and, in spite of older reports of the preparation of non-protein antibody solutions, present evidence justifies the current belief that *antibodies are modified serum globulin.* The physical and chemical properties of antibodies are, then, those of globulin. More precise knowledge is obviously directly dependent upon the state of knowledge of protein chemistry, whose present unsatisfactory nature

[66] Northrup: Proc. Amer. Phil. Soc., 1941, 85:13.

is indicated by the feeling on the part of a number of competent investigators
that the serum proteins, including globulin, do not exist as such in the body
but are artifacts arising as a consequence of laboratory manipulation.

If antibodies are to be regarded as modified serum globulin, the nature
of the modification, *i.e.*, the differences between immune and normal serum
globulin, is of considerable interest. Unfortunately these differences are ex-
pressed chiefly in the ability of the modified globulin to combine with the
antigen, for, in spite of intensive study, there appear to be no consistent
differences in physical and chemical properties between the two (see p. 313).
Exception must be made in the case of resistance to the action of proteolytic
enzymes; the immune globulin appears to be considerably the more resistant
to hydrolysis with pepsin in acid solution, and partial purification of antitoxin
by this means has been generally used in its commercial preparation.[67]

Antibody Formation. The Breinl-Haurowitz theory[68] of antibody formation
postulates that some antigen remains within the antibody-forming cell to pro-
vide a template or pattern for the synthesis of specific antibody. On this basis
Pauling[69] proposed, on purely hypothetical grounds, a theory of the structure
and process of formation of antibody. He has suggested that if the normal
globulin molecule is formed by the coiling of peptide chains after their syn-
thesis, the surface character of the molecule could be determined in the
process of coiling. In the presence of the antigen template the peptide chain is
coiled in such a way as to give a surface pattern of intermolecular forces com-
plementary to the pattern of the determinant group of the antigen molecule
(see p. 281). Dissociation of the antigen-antibody complex frees the antigen
to act as a template for the formation of another antibody molecule, and so on.
Immune globulin would, therefore, differ from normal globulin only with
respect to the manner of coiling of the peptide chains.

This theory is susceptible to test, for normal globulin may be denatured
slowly by heat, alkali, etc., to uncoil the peptide chains; then if it is renatured
in the presence of the antigen, the recoiling should be oriented with respect
to the antigen. This kind of experiment has been carried out by Pauling and
Campbell[70] who denatured ox γ-globulin by heating to 57° C. for eleven to
fourteen days in the presence of 1,3-dihydroxy 2,4,6-tri(*p*-azophenylarsonic
acid) benzene or pneumococcus Type 3 polysaccharide. After removal of the
"antigen," the globulins gave specific precipitin reactions and in the second
instance specifically agglutinated Type 3 pneumococcus but only in very low
dilution. These observations provide strong support for the theory and are
especially notable because they are the first demonstration of antibody forma-
tion *in vitro*. The cellular origin of antibody is discussed elsewhere (p. 323).

Consistent with the intimate relation of antibody and serum globulin is the
increase in serum globulin, especially γ-globulin, on immunization. This in-
crease in general appears to take place at the expense of serum albumin, which
is decreased, but in some cases the reduction in albumin does not occur and

[67] See the general review of the action of enzymes on antibodies by Stack: Bull. Hyg.,
1947, 22:733.
[68] Breinl and Haurowitz: Ztschr. f. physiol. Chem., 1930, *192*:45.
[69] Pauling: Jour. Amer. Chem. Soc., 1940, *62*:2643.
[70] Pauling and Campbell: Jour. Exp. Med., 1942, 76:211.

the total serum protein increases to as much as 15 per cent. Not all of the new globulin is antibody, however, for less than one-half is capable of reacting with antigen. It is of some interest that large and small antibody molecules are produced, depending upon the animal species immunized; those from the horse, cow and pig are large with molecular weights of 910,000 to 930,000, while those formed by man, monkey and rabbit are considerably smaller with molecular weights of 156,000 to 196,000.[71]

The "Unitarian" Hypothesis. It was originally supposed by Ehrlich that the various demonstrable antibodies—antitoxins, agglutinins, precipitins and the like—are separate, distinct and independent substances, a concept that is apparently supported by discrepancies in the titer of one antibody and that of another in a given immune serum. The essential similarity of the agglutination and precipitation reactions was, however, early apparent, and with increasing knowledge it has become clear not only that it is unnecessary to postulate a multiplicity of antibodies but that the weight of experimental evidence suggests that a pure antigen stimulates the formation of but a single antibody. This essential identity of the various antibodies has been urged by a number of workers, and by Zinsser[72] in particular, in what is known as the *unitarian hypothesis.*

The basic feature common to all the immunological reactions is the union of antigen and antibody and, according to the unitarian hypothesis, this antibody is the same regardless of the consequences of union, which are, of course, variable and dependent upon the nature of the antigen and the conditions under which the reaction takes place. After union with antibody the antigen is sensitized to the lytic action of complement, is able to fix complement, is rendered susceptible to phagocytosis or is agglutinated or precipitated. The outcome is a consequence of the physical state of the antigen, *i.e.,* whether it is cellular, particulate or in solution, and of the particular consequences of union the experiment is designed to show, *i.e.,* whether the test is one of complement fixation, agglutination, etc. Instances of this have already been noted, such as the precipitation of toxin by antitoxin in the Ramon flocculation, the agglutination of antigen-coated particles by precipitating sera, the fixation of complement by precipitates from the precipitin reaction and hemagglutination in the absence of complement.

In a very real sense, however, the antibody is not a single substance since the combining site or sites on the individual globulin molecules may be well or poorly developed. This is expressed as ability to combine with the homologous antigen and is usually referred to as "avidity." Antibody may be separated by appropriate absorption technics into fractions of varying avidity; the less avid fractions are regarded as poorly developed or incomplete antibody. The differences are of degree rather than kind, of course, and do not invalidate the unitarian theory.

The unitarian concept postulates only that each antigen gives rise to a single antibody; a multiple antigen such as a bacterial cell will, of course, stimulate the formation of a series of antibodies, one for each of the immunologically active constituents of the cell. Discrepancies in antibody titer of antibacterial

[71] *Cf.* Kabat: Jour. Exp. Med., 1939, 69:103.
[72] *Cf.* Zinsser: Jour. Immunol., 1921, 6:289.

sera may, then, appear without invalidating the concept of the unity of anti-
bodies; the flagellar agglutinins for the typhoid bacillus, for example, may have
quite a different titer from that of precipitins for the cell substance. Similar
discrepancies in complement-fixing and precipitin titers of antisera for pure
antigens, such as egg albumin, in which only a single antibody is concerned,
are explained by the fact that antigen-antibody ratios optimal for complement
fixation are not optimal for precipitation.

The evidence for the essential identity of what were at first regarded as dis-
tinct antibodies for a single antigen is indeed impressive, and the unitarian
hypothesis may be regarded as substantially in accord with the facts.

THE ANTIGEN-ANTIBODY REACTION

The mechanism of the antigen-antibody reaction has already been touched
upon briefly in the preceding sections. The reaction between these two sub-
stances may be considered here, however, at somewhat more length and in
general terms.

It will already be apparent that the antigen-antibody reaction appears to take
place in two separate and distinct stages, the first in which the two combine
and the second in which the consequences of that union appear as agglutina-
tion, precipitation and the like. In mixtures the two processes undoubtedly go
on at the same time; it is not necessary that the first proceed to completion
before the second may be initiated. Direct evidence of such a two-step reaction
is found in the union of antibody with simple haptenes, in which the former
is saturated and unable to react further with complete antigen even though
no second stage is observable. The theoretical aspects of this union are con-
cerned not only with the actual mechanism of union but also with the at-
tributes of antigen and antibody that make possible their reaction with one
another.

The only comprehensive formulation of the theoretical aspects of immu-
nology is that of Ehrlich,[73] a formulation which has constituted the theoretical
basis upon which a vast amount of experimental work has rested. Let us con-
sider Ehrlich's basic concept, first as it was developed, and second in the light
of present knowledge.

Ehrlich's Receptor Theory. Ehrlich's attempt to develop a theoretical
basis for the explanation of the immunological phenomena has been touched
upon briefly in connection with the antitoxins. The theory he developed, how-
ever, is not confined to the toxin-antitoxin reaction but is a general one of im-
munity in the broad sense.

The receptor theory rests upon the basic assumption that the various cells of
the animal body obtain their nutriment from the blood or lymph in which they
are bathed through the agency of localized cell substances, the *cell receptors,*
which have combining affinities with food substances. The receptors may be re-
garded as bearing the same relation to the main body of the cell (*Leistungs-
kern*) that the side chains of complex molecules bear to the central molecular
nucleus; hence the receptor theory is sometimes known as the *side chain theory.*
These receptors may be of simple constitution, adapted to the taking up of
relatively simple substances, or they may be highly complex and capable of

[73] Ehrlich: *Studies on Immunity.* New York. 1906.

anchoring large and complex protein molecules. Each cell may, of course, contain a large number of receptors of different affinities and degrees of complexity.

It is plausible to assume that when bacteria or other alien cells or their products are introduced into the body, the combining affinities of certain receptors may be satisfied by bacterial substances just as by similarly constituted food molecules. The anchoring of toxic substances, however, unlike that of food substances, is followed by damage to the cell and loss of the particular side chain or receptor that unites with the toxic element. When injury to the main body of the cell is not carried too far, repair can take place and the receptors be regenerated. There is a tendency in the regeneration of lost parts, in this case receptors, to over-compensate, and the free receptors formed in excess of the needs of the cell are discharged into the blood stream. *These free receptors are the antibodies*, the antitoxins, agglutinins, lysins and the like. They differ in complexity. The simplest, or receptors of the first order, are those which combine with toxin and, when free, constitute antitoxin; receptors of the second order are somewhat more complex and are functional in agglutination and precipitation; receptors of the third order are still more complex and function in the lytic reactions in which complement plays a part.

Similar representations of the antigenic substances and of complement necessarily follow in this concept of the nature of the immune bodies. Toxin, for example, is assumed to be a relatively simple substance with two functional parts, a haptophore which unites with the receptor and a toxophore which exerts the poisonous effect of the substance. In this terminology, toxoid would be regarded as toxin in which the toxophore portion is inactivated or destroyed.

The mechanism of the reaction of the antibodies with their respective antigens is apparent from the foregoing considerations. The union of toxin and antitoxin is the simplest type, the haptophore group uniting specifically with the corresponding portion of the free receptor or antitoxin molecule and the toxophore group being neutralized in that it is prevented from exerting its action on the cell. Agglutination is much the same so far as the union of antigen and antibody is concerned, but the presence of the zymophore group brings about clumping. In the case of complement fixation it is assumed that the receptor of the third order, after breaking off, functions as a sort of bridge or intermediary body (*Zwischenkörper*) which unites with complement at one end and with antigen at the other, thus sensitizing the antigen to the lytic action of complement.

It will be clear that, in its simplest terms, the receptor theory is a plausible explanation of immunological phenomena. There is evidence, for example, that the various bacterial toxins are bound in each case to particular cells of the organism. The tetanus toxin, when mixed *in vitro* with emulsions of fresh organs, manifests an affinity for different organs in different animals. In man, the horse and the guinea pig, only the central nervous system is able to bind the toxin, a finding in complete accord with the pathology of tetanus. If a mixture of tetanus toxin and guinea-pig-brain emulsion in suitable proportions is injected into a susceptible animal, the animal is entirely unaffected, just as if tetanus antitoxin (free receptors) had been used in place of fresh cell substance (cell receptors). Other phenomena are equally well explained in terms

Immunity

of this theory. It will be recalled, for example, that complement was early separated into two parts, mid-piece and end-piece, a finding in keeping with the postulation of zymophore and haptophore portions of this substance.

Certain inadequacies of the receptor theory were apparent almost from the beginning, however, since new knowledge, rather than being predicted by the theory, necessitated various modifications. These discrepancies arose, for the most part, in connection with the mechanism of the first stage of the antigen-antibody reaction and, to some extent, with that of the second stage. It will be recalled that the toxin-antitoxin reaction, for example, was regarded by Ehrlich as analogous to the neutralization of a strong acid by a strong base, a concept that was brought into harmony with experimental observation through the postulation of components of toxin of varying degrees of avidity for antitoxin. Similar modifications and additions, which cannot be considered in detail here, resulted in the course of time in an unwieldly structure that became obviously unsatisfactory.

It may be noted here that the concept of Arrhenius and Madsen of the antigen-antibody reaction as analogous to the neutralization of a weak acid by a weak base was, in essence, an attempt to retain much of the receptor theory and at the same time account for the Danysz phenomenon and related observations as well as the dissociation of antigen-antibody complexes.

The Antigen-Antibody Reaction as an Adsorption Phenomenon. In many respects the antigen and antibody solutions are best regarded as colloidal systems and the union of these substances as adsorption phenomena, physical in nature rather than chemical. This interpretation has been strongly urged by Bordet[74] and others. Stripped to its essentials, such an interpretation means that the Freundlich isotherm[75] describes the antigen-antibody reaction with a reasonable degree of accuracy. The successful application of this concept to the first stage of the antigen-antibody reaction is well illustrated in the case of the toxin-antitoxin reaction which has been discussed in this connection in an earlier section. Similarly, the clumping of bacteria under the influence of immune serum, an example of the second stage of the reaction, responds equally well to such an interpretation, as has been pointed out before.

The course of events in the antigen-antibody reaction, as seen from this point of view, may be summarized briefly: there is first a specific adsorption of the immune globulin on the surface of the antigen. The cell or protein molecule thus sensitized behaves, not as the unaltered antigen, but as a particle of serum globulin. The aggregation of the coated particles in the precipitin or agglutination reactions is, then, nothing more than the precipitation of this altered colloid by salt. The sensitized antigen is also capable of adsorbing complement whose fixation may or may not be followed by lysis as the nature of the antigen permits. The evidence for some such mechanism is very strong indeed, and it is highly probable that certain phases of the antigen-antibody reactions are essentially manifestations of the properties of colloidal systems. This ines-

[74] Bordet: *L'Immunité.* Paris. 1920.
[75] The relation between the amount of a substance adsorbed and that remaining in solution was given by Freundlich as x/m = aC^n, when x is the amount absorbed, m, the surface on which it is adsorbed, C the final concentration of the substance in solution and a and n are constants.

capable conclusion led many to abandon the receptor theory as entirely out-moded.[76]

This description of the immune phenomena is by no means entirely satis-factory, however, its most glaring deficiency being its inability to account for specificity. While the union of antigen and antibody is, in all probability, an adsorption reaction, why typhoid antibody is adsorbed on the typhoid bacillus and at the same time not on the dysentery bacillus is unexplained. In addition to this failure to account for immunological specificity, certain minor dis-crepancies may be observed; there is, for example, agglutination or precipitation over a relatively wide pH range (in the presence of electrolyte) instead of a marked optimum at the isoelectric point of serum globulin, as might be ex-pected. The adsorption isotherm, furthermore, does not approach an asymptote, and the antigen-antibody reaction shows wide variations from expected values at high concentrations of either reagent. These and other minor points are, however, probably of no great significance. In general, it may be said that al-though this adsorption theory is thoroughly satisfactory in many respects, it accounts only for some, by no means all, of the immunological phenomena.

The Modern Concept. As might be expected, the modern concept of the mechanism of the antigen-antibody reaction is an outgrowth of the theories of Ehrlich and Bordet. The reacting substances are regarded as colloids whose union lies in the realm of surface chemistry and is in part physical and in part chemical. For purely technical reasons, the observations which have led to this concept have been made on the precipitin reaction for the most part; a discussion of the present position is, then, best carried on in terms of this reaction.

It is already apparent from the earlier discussion of antigens that immuno-logical specificity may be conferred by a relatively small prosthetic group or haptene attached to the large protein molecule. Furthermore, it was pointed out that similarities in the structure and configuration of these determinant groups are causally related to the immunological behavior of synthetic antigens. Immunological behavior means, of course, the ability both to provoke the forma-tion of antibodies and to react with them. The significance of these observations on immunological specificity to the nature of the antigen-antibody reaction will be apparent at this point; the molecular structure of the prosthetic group determines whether or not the antigen and antibody will react.

There is good experimental evidence, too detailed and extensive to be con-sidered here,[77] that the union of antigen and antibody is an adsorption in which a particular molecular structure is adsorbed with a high degree of specificity. It is probable that there is no chemical union (many of the de-terminant groups are not characterized by chemically active radicles) but that the adsorption is a consequence of the operation of intermolecular forces (sec-ondary valencies) whose specificity lies in the nature of the field of force arising from the arrangement of the atoms within the determinant group. The

[76] Cf. Manwaring: Jour. Immunol., 1926, 12:177; also a chapter by the same writer in Jordan and Falk: *Newer Knowledge of Bacteriology and Immunology*. University of Chicago Press, Chicago. 1928.

[77] Cf. Marrack: *The Chemistry of Antigens and Antibodies*. Medical Research Council Special Report Series No. 230. 1939. Also Heidelberger: Bact. Rev. 1939, 3:49.

nature of these forces is not altogether clear. In this connection it is of particular interest that Rothen[78] has claimed, by the use of monomolecular films, that the antigen-antibody reaction occurs over distances as great as 200 Å, considerably greater than that calculated for small molecules. It is to be noted that the functional structure of the antigen is directly known; that a corresponding field of force, of such spatial distribution that corresponding active points of antigen and antibody can come into apposition simultaneously, is possessed by the antibody is indirectly established by the fact that the two combine and by the structure of the determinant groups of antigens which show cross-reactions. It may be pointed out here that radicles that are mutually replaceable in crystals are immunologically equivalent. The formation of mixed crystals is, however, the severer test, for here all dimensions must fit with a high degree of precision, while in the combination of antigen and antibody only two dimensions need be oriented.

The alteration in normal serum globulin which converts it to antibody is, then, an alteration in the arrangement of polar forces on the surface of the molecule. It is probable that the surface of the normal globulin molecule presents a mosaic of groups of varying polarity and that some areas are well adapted to binding certain molecules. It is plausible to assume that relatively slight alterations in the structure of the globulin molecule give rise to areas with affinities for considerable areas of certain determinant groups, alterations so slight as to be undetectable by the usual means (see p. 308).

Valence of Antigen and Antibody. Both antigen and antibody show evidence of heterogeneity with respect to their reaction with one another. A given preparation of an antigen, such as pneumococcus polysaccharide, contains some antigen that will not combine with antibody, and artificial antigens contain only an average number of reactive groups per molecule, some of which may have too few to combine. Similarly, the avidity of antibody is variable, the more avid molecules combining rapidly with antigen while the less avid ones combine much more slowly. Antigen and antibody may, then, coexist without union if they are so-called modified antigen and weak antibody. It is not definitely known what differences are reflected by this behavior; possibly incompletely developed adsorption sites occur.

The valence of both antigen and antibody has been of some interest, especially in connection with the nature of the second stage of the antigen-antibody reaction. By analysis of antigen-antibody precipitates,[79] for example, for arsenic when arsenic-containing artificial antigens are used, or for total nitrogen in the case of polysaccharide-antibody complex, it is possible to determine the maximum number of antibody molecules which combine with a single molecule of antigen. The valence of the antigen appears to be a function of the surface area of the molecule; the functional valence of ovalbumin of a molecular weight of 40,500 is about 5 to 6, while that of thyroglobulin of 650,000 is 30 to 40. These are, of course, minimal values. Maximum values are readily obtained in the case of artificial antigens and it has been found that 10 to 20 active groups must be introduced into the molecule. Not all of such a maximum number of active groups are functional

[78] Rothen: Jour. Biol. Chem., 1947, *168*:75.
[79] See the review by Heidelberger: Bact. Rev., 1939, *3*:49.

in the reaction with antibody, for when they are too close together the antibody molecules get in one another's way to prevent complete utilization, a phenomenon termed *steric hindrance.*

The valence of the antibody molecule has been the subject of some discussion which cannot be considered at length here. Suffice it to say that, while some workers believe that antibody is regularly univalent, it is generally believed that present evidence indicates that it is usually divalent. Currently accepted theories of antibody formation include the assumption of divalency. Univalent or "incomplete" antibody is known to occur, however, and possibly is represented by the "weak" antibody noted above.
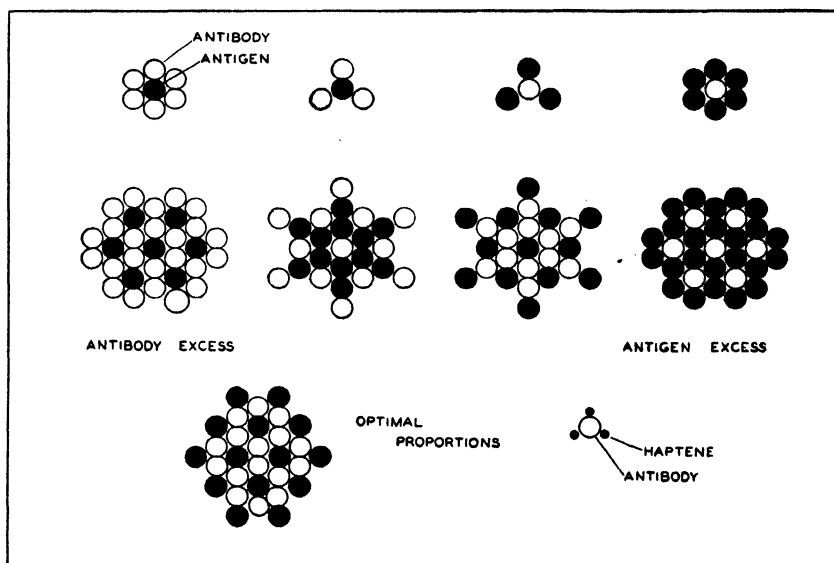
Fig. 38. Diagrammatic representation of possible arrangements of antigen and antibody molecules in the complex. Modified from Marrack.[77]

The Second Stage of the Antigen-Antibody Reaction. The second stage of the antigen-antibody reaction is generally regarded as an extension of the process of union and therefore specific, rather than a separate and non-specific process. The specific absorption involves, of course, the apposition of polar groups as indicated above. Thus, if the antibody is divalent it can combine with two molecules of multivalent antigen, the antigen with additional antibody, etc., to form a mesh-like arrangement or lattice structure as illustrated in Fig. 38, large aggregates of which settle out as the antigen-antibody precipitate. This is the *lattice hypothesis* of Marrack.

This theory is dependent, of course, on the existence of at least divalent antibody and it is for this reason that the valency of antibody has been of particular interest. As indicated above, present evidence strongly supports its multivalency. Thus, if increasing amounts of antigen are added to a constant amount of antibody, three zones may be distinguished: first, a zone of antibody excess in which uncombined antibody is present; second,

an equivalence zone in which both antigen and antibody are completely precipitated; and, third, a zone of antigen excess in which uncombined antigen is present. The proportions of antigen and antibody in the precipitate vary with the initial concentrations of the reaction substances and behave as if they were multivalent with respect to one another, the observed molecular ratios being consistent with the assumption of divalent antibody. Similarly, the "incomplete" or monovalent antibody described by Heidelberger and Mayer[80] combined with antigen but did not give a precipitate, and Pauling and his co-workers[81] have shown that synthetic antigens containing two or more haptenic groups per molecule would form a precipitate with antibody, while those containing only one haptenic group would not. Evidence of this sort, of which there is a good deal,[82] strongly supports the lattice hypothesis.

It has been urged by some, especially Eagle[83] and Boyd[84] in recent years, that precipitation is the non-specific result of neutralization or blocking off the polar groups of the complex; if the polar groups remaining on the free surface are not sufficient to keep the complex in solution, it behaves as a hydrophobic colloid and is precipitated by electrolyte. The evidence does not appear to support this view.

A number of theories of the interaction of antigen and antibody have been proposed. Heidelberger[85] has viewed it as a chemical reaction between substances multivalent with respect to one another according to the law of mass action, resulting in a series of bimolecular reactions. The initial reaction between antigen (S) and antibody (A) is:

$$A + S \rightleftharpoons AS$$
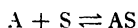
and, for instance, in the region of excess antibody:

$$AS + A \rightleftharpoons AS.A$$
$$AS + AS \rightleftharpoons AS.AS$$

and so on, with the formation of visible aggregates with the formula $(AS)_x$, or more properly $(A_nS_m)_x$ since the ratio of antibody to antigen is greater than unity, probably more than 2, in the equivalence zone. Hershey[86] has similarly developed a theory that allows the calculation of equilibrium constants which agree well with observed values. Teorell's[87] theory is based on the assumption that the reaction is similar to the dissociation of a polybasic acid where monovalent antibody (A) corresponds to the hydrogen ion, and polyvalent antigen (G) to the anion. Interaction would result in the formation of a series of compounds, A_nG, $A_{n-1}G$, ...AG, and assumptions

[80] Heidelberger and Mayer: Jour. Bact., 1940, 39:37.

[81] Pauling, Pressman, Campbell, Ikeda and Ikawa: Jour. Amer. Chem. Soc., 1942, 64:2994.

[82] Reviewed by Pauling, Campbell and Pressman: Physiol. Rev., 1943, 23:203.

[83] Eagle: Jour. Immunol., 1930, *18*:393.

[84] Boyd: Jour. Exp. Med., 1942, 75:407.

[85] Heidelberger: *Relation of Proteins to Immunity*. Chapter XVII in Schmidt: *Chemistry of the Amino Acids and Proteins.* Charles C Thomas, Springfield, Ill. 1938.

[86] Hershey: Jour. Immunol., 1941, 42:455; *ibid.*, 1942, 45:39.

[87] Teorell: Jour. Hyg., 1946, 44:227, 237.

made as to the solubilities of the reaction products. He has shown that anti-gen-antibody precipitates correspond well with the calculated values.

The extent to which the indicated mechanism of the precipitin reaction may be generalized to include other manifestations of the antigen-antibody reaction is not yet altogether clear. There are many indications, however, that the process of union is essentially the same in all cases, though final proof is lacking. There is as yet no answer to such questions as that of the manner in which a red blood cell is rendered susceptible to the lytic action of complement or that of the nature of the process of toxin neutralization.

The relation of the modern concept of the antigen-antibody reaction to the older theories will be readily apparent. It may be pointed out that Ehrlich's concept of a chemical basis of specificity has been borne out; haptophores and receptors are, in present terminology, determinant radicles and adsorption sites. The mechanism of union, however, conforms to Bordet's adsorption hypothesis, and what were once regarded as mutually exclusive theories have been found to be but two different aspects of the immuno-logical reactions.

IMMUNITY—THE IMMUNE STATE

The foregoing discussion of antigens, antibodies and the antigen-antibody reactions is, essentially, a consideration of the various components of that state of increased specific resistance designated as immunity. The question arises, then, as to what extent these component parts represent the immune state. Can they be put back together to yield a satisfactory account of this phenomenon? In general, it may be said that such a synthesis does result in a relatively complete picture; it is certainly far more satisfactory than corresponding attempts to analyze virulence and non-specific resistance.

It will be clear from preceding considerations that two kinds of activity, closely interrelated, are operative in immunity; the antibodies, present in the body fluids, function in the first case, and the body cells, the phagocytes, in the second. The two are not infrequently separated, the activity of antibodies constituting the subject matter of *humoral immunity* and that of the phagocytic cells making up the subject of *cellular immunity*.

Humoral Immunity. Since the appearance of antibodies follows recovery from many of the infectious diseases, it is tempting to assume that these substances are causally related to the observed state of increased resistance. Observation and experimental evidence have amply justified this assumption, obviously in some instances and in a more subtle fashion in others.

An antibody whose association with the immune state is direct and clear is antitoxin. The toxin produced by the invading microorganism has an affinity for the cells as well as antitoxin present in the body fluids, and the protective effect of circulating antibody must, then, be due in part to its superior avidity for the toxin. Such superior avidity generally, but not always, exists. Instances are known in which the toxin unites with the tissue substance in preference to combining with the antitoxin in the blood. The affinity of the cell substance for the toxin is not a constant quality, but fluctuates under different conditions, notably in the upper limits of active immunization. The tissues of an animal treated with increasing doses of toxin sometimes become hypersensitive to the action of the toxin and, in spite of the fact that large quantities of antitoxin are circulating in the blood, the toxin combines by preference with the tissue substance and causes the death of the animal. Variation in the avidity of circulating antitoxin has been pointed out above. In general, however, the presence of circulating antitoxin is the determining factor; for example, it is not necessary that antibodies to the bacterial cell substance be present in an individual immune to diphtheria or tetanus. The skin tests for immunity, the Schick test for immunity to diphtheria and the Dick test for immunity to scarlet fever, are

tests for the presence of antitoxin; the toxin is injected intradermally and, if not neutralized, gives rise to a local reaction. Such an antitoxic immunity is an immunity to the disease, but not to infection with, for example, diphtheria bacilli or streptococci, and the carrier state is quite as common in the immune as in the non-immune.

The presence of the bactericidal and lytic antibodies is clearly of considerable advantage to the host and these substances are also an obvious part of the defense mechanism. Similarly, the inhibition of multiplication of trypanosomes is an important part of the defense against certain species such as *Trypanosoma lewisi* (p. 769). Agglutinins and precipitins would appear on the surface to serve no useful purpose in the defenses of the immune animal; for example, bacteria are not killed by agglutination. The clumping of bacteria and the precipitation of soluble foreign protein, however, considerably facilitate the process of phagocytosis in that many bacteria may be engulfed at one time and insoluble masses of precipitated foreign protein may thus be removed.[1] In the case of pneumococcus antisera, for example, protective antibody as measured by mouse assay appears to be identical with precipitin and agglutinin. The pneumococcus antibody has been found to enter pneumonic lesions in rats, agglutinating the free pneumococci in the alveoli and thus stopping the spread of the infection.[2] Furthermore, heterologous protection may be demonstrated when portions of the antigenic mosaic are shared.[3] Not all antibodies demonstrable by *in vitro* antigen-antibody reactions have protective qualities, however; in the case of the enteric bacilli protection appears to be associated with antibody to the heat-stable somatic antigens. It may be noted that in one instance there is evidence that precipitins play a direct part in defense; it has been found[4] that the larvae of *Nippostrongylus muris* are stunted and immobilized in the skin of the immune animal with the formation of precipitates in and around them, and a similar reaction may be observed *in vitro*. In general, however, the precipitins and agglutinins function as a part of the defensive scheme in conjunction with the process of phagocytosis.

The importance of phagocytosis in resistance to infection is very great indeed and, in consequence, the opsonins assume particular importance from the theoretical point of view. In practice, though, opsonin titer is generally low as compared to antitoxin and other antibody titers and is highly variable; the value of the opsonic index as a measure of immunity is not great, possibly because of the use of polymorphonuclear leucocytes in *in vitro* tests. Opsonic activity, of course, involves the direct cooperation of certain cellular elements of the body, and any consideration of these antibodies, as well as agglutinins and precipitins, forces consideration of the cellular defenses and indicates the futility of any attempt to differentiate sharply between humoral and cellular immunity.

The implications of antitoxic immunity in diphtheria, tetanus and scarlet

[1] *Cf.* Cannon: Physiol. Rev., 1940, 20:89.
[2] Wood: Jour. Exp. Med., 1941, 73:201.
[3] See the studies on the typhoid bacillus by Luippold: Amer. Jour. Hyg., 1942, 36:354.
[4] Sarles and Taliaferro: Jour. Inf. Dis., 1936, 59:207; Taliaferro and Sarles: Jour. Inf. Dis., 1939, 64:157.

fever noted above may be carried somewhat further. It is clear that the immunity so produced is what may be called an *effective immunity* in that it is an immunity against the infection or the disease produced by pathogenic bacteria. In addition to antitoxin, the antibodies to the various antigenic components of the bacterial cell may vary widely in their contribution to such an effective immunity. Thus, protection against pneumococcus infection is associated for the most part with antibody to the type-specific capsular antigen, while that to the somatic antigen contributes to only a relatively minor extent. On the other hand, in the case of enteric bacilli and the cholera vibrio effective immunity is produced by immunization with somatic or O antigen, and antibody to the flagellar antigenic complex has little protective action.

In certain instances the O antigen may be broken down somewhat further with respect to effective immunity. As indicated elsewhere (p. 452), the typhoid bacillus exists in two forms, the V form which contains Vi antigen, and the W form which does not. Vi-containing strains which lack typhoid bacillus O antigen can be obtained by dissociation, and certain other bacteria, notably a widely used strain of *Salmonella ballerup,* contain Vi antigen but are unrelated to the typhoid bacillus with regard to the specificity of the O antigen. Immunization with Vi-containing bacteria which lack the typhoid bacillus O antigen, whether typhoid bacilli or not, will protect against infection with V strains of the typhoid bacillus, but not with W strains. Conversely, immunization with a W strain of the typhoid bacillus will protect against infection with either V or W forms of this organism, but not against infection with Vi-containing bacteria that lack the O antigen of the infecting strain. It is concluded, therefore, that antibody to the O antigen and that to the Vi antigen are concerned in protection. The components of the other O antigenic complexes occurring in the Salmonella group (p. 433) are not sharply differentiated in that all seem to contribute to an effective immunity. The antigens so related to the effective immune response are sometimes spoken of as "essential immunizing antigens." When they are shared by related bacteria, such as Salmonellas, or phylogenetically unrelated microorganisms (p. 328), a cross immunity results, but when they are not shared by even closely related bacteria such as the various types of pneumococci, the cross immunity is of a low order. It follows, of course, that not only should an effective immunizing preparation contain the significant antigens, but antigens extraneous to the development of an effective immunity may be omitted. The first consideration has indicated the use of typhoid vaccine prepared from Vi-containing strains, and the second is the basis of the endotoxoid vaccines such as those prepared from the plague bacillus.

The extent of cross immunity also determines the polyvalency of vaccines, *i.e.,* the number of bacterial strains which enter into its preparation. This is of obvious practical significance as in the preparation of dysentery vaccines. Such vaccines are usually quite toxic and if they must be made highly polyvalent, each additional strain or type of dysentery bacillus contributes its toxicity, with the result that the toxicity of the preparation increases if the amount and therefore antigenicity of the components are not reduced.

Cellular Immunity. The role of phagocytic cells in resistance to infec-

tion was established by Metchnikoff[5] on the basis of studies on lower forms such as the water flea, Daphnia. He distinguished two types of cells, the *microphages* or polymorphonuclear leucocytes (heterophils) and the *macrophages* or large mononuclear cells occurring both free in the blood stream and fixed as tissue cells. The former were regarded as of prime importance in the bacterial infections, while the macrophages were thought to be only indirectly active except in chronic infections such as tuberculosis and leprosy. With advances in knowledge, however, it has become clear that although their immediate mobilization accentuates the limited significance of the polymorphonuclear leucocytes, the variety of fixed and free connective tissue cells of mesenchymal origin, grouped under the general head of macrophage, is of considerably greater importance in resistance to disease.

The distribution and interrelationships of these phagocytic cells may be considered briefly in outline form:[6]

A. The predominantly fixed cells of the reticular and loose connective tissue which can be divided into two great groups:
(1) fixed and free macrophages, including the reticular cells, and
(2) the fibroblasts of connective tissue and the endothelial cells lining the ordinary blood vessels.

(1) Macrophage is essentially a physiological designation for almost any large mononuclear connective tissue cell which is predominantly phagocytic and includes
(a) fixed cells such as
1. *pericytes* (Maximov), fixed, undifferentiated outstretched cells in the adventitia of all the small blood vessels of loose connective tissue throughout the body.
2. *reticular cells* which, with fibers, form the stroma of all reticular (myeloid and lymphatic) tissues, and
3. *littoral cells* (Siegmund) which line the sinuses of the reticular tissues, liver, hypophysis and adrenal. In the liver these are designated as *Kupffer cells*. Although frequently called endothelial cells or cells of the special endothelium, these are in fact either true reticular cells or have greater developmental potencies than ordinary endothelial cells.
These cells can divide by mitosis, become phagocytic, and develop into fibroblasts or practically any other blood or connective tissue cell. Here it is important that they can become phagocytic either in their fixed position (fixed macrophages) or after rounding up and becoming free (free macrophages).
(b) The free cells occurring in the loose connective tissue are variously known as *histiocytes, clasmatocytes, rhagiocrine cells* or wandering resting cells, are either phagocytic or can become so without morphological change, can reproduce by mitosis, and transform into fibroblasts.
(2) The fibroblasts and endothelial cells are morphologically characterized by outstretched, ill-defined cytoplasm, a large oval vesicular nucleus containing dust-like chromatin granules, and small nucleoli.
(a) The fibroblasts divide by mitosis but do not develop into other cells (except in bone and cartilage) and are rarely phagocytic although instrumental in repair and walling off foreign material.
(b) The endothelial cells which line the larger blood vessels and capillaries (not including the littoral cells) likewise are rarely phagocytic but may be transformed into fibroblasts.

[5] Metchnikoff: *Immunity*. Cambridge. 1905.
[6] This material is taken, with only minor modifications, from Taliaferro: *The Immunology of the Parasitic Protozoa*. Chapter XVIII in Calkin's *Protozoa and Biological Research*. Columbia University Press, New York. 1941.

B. The free connective tissue and blood cells.
(1) The cells of the blood and lymph are classified as to whether of myeloid or lymphoid origin:
    (*a*) The *lymphoid cells* of the blood include the various sized lymphocytes which, together with monocytes, are termed *agranulocytes*. They divide mitotically and can develop into macrophages with all the latter's developmental potencies. As they become transformed into macrophages, they show increased amounts of cytoplasm, their nuclei take on macrophage characteristics and they become phagocytic. These transitional forms are known as *polyblasts*.
    (*b*) The *myeloid cells* are the various *granulocytes* (heterophils or polymorphonuclears, eosinophils and basophils), the erythrocytes and platelets. Of these the heterophils are functional in immunity by virtue of their phagocytic activity but are "end" cells which do not reproduce or develop into other cells.
(2) The free mesenchymal cells are lymphoid cells indistinguishable from lymphocytes which occur in varying numbers in reticular and loose connective tissue and here act as precursors or "stem" cells of lymphoid and myeloid cells and hence may give rise to macrophages. They are variously termed *lymphocytes, hemocytoblasts, lymphoblasts, myeloblasts* or *monoblasts* according to varying theories of blood formation. Under normal conditions lymphocytes in lymphatic tissue give rise only to lymphocytes and hemocytoblasts in bone marrow only to myeloid cells but under abnormal stimuli they may exhibit their full potencies for development. These stem cells are self-perpetuating, but as noted above, may arise from the fixed mesenchymal cells.

The Systems of Cells. It will be apparent that the cells primarily associated with defense against invading microorganisms are widely distributed through the body in the blood and lymph, cartilage, bone, reticular (blood-forming) tissue of the myeloid and lymphatic organs and in the loose connective tissue associated with the skin, omentum, liver, lung, etc. It is customary to group the macrophages into so-called systems of cells, the best known of which is Aschoff's[7] *reticulo-endothelial system.* The term is not a good one, however, for, as indicated above, the cells of the endothelium proper are not phagocytic; although called endothelial cells, the phagocytic littoral cells (such as Kupffer cells) are similar to or identical with reticular cells. Perhaps a better designation is simply *macrophage system* or the broader term *lymphoid-macrophage system,*[8] which includes the lymphoid cells and the transitional polyblasts.

Local Defense. The cellular response to an inflammatory stimulus[9] is characterized by an initial migration of polymorphonuclear leucocytes to the point of injury. These cells are not numerous and soon disappear when the inflammatory material is sterile, but when bacteria are present they continue to migrate from the blood vessels and actively phagocytose the invading microorganisms. The functions of this first line of defense, although important, are strictly limited, since the cells are short-lived and do not multiply *in situ* but must be continuously recruited from the blood stream.

More important to local defense are the lymphoid cells of the blood and lymph, the lymphocytes and monocytes, which also migrate from the blood vessels but which, unlike the polymorphonuclear leucocytes, are long-lived

[7] Aschoff: Ergebn. inn. Med. Kinderheilk., 1924, 26:1.
[8] Taliaferro and Mulligan: Indian Medical Research Memoir No. 29, 1937.
[9] *Cf.* Menkin: *The Dynamics of Inflammation.* The Macmillan Company, New York. 1940.

and multiply in the tissues. These stem cells may be transformed to macrophages which, together with macrophages already present in the area, actively phagocytose and digest the invading microorganisms. When large bodies of foreign material are present, the macrophages may fuse to form foreign body giant cells; when microorganisms are indigestible, they may form giant cells around them such as the epithelioid cells of the tubercle. Their progressive development into fibroblasts supplies the active elements for regeneration and repair, the formation of scar tissue and the walling off of foreign bodies.

Certain other cells may take part in the late stages of the inflammatory process; the eosinophils, for example, may play a part in the detoxification of proteins and their disintegration products.

General Defense. When a stimulant is distributed over a large part of the body the reaction is regarded as a general rather than local one; such distribution, however, usually means that the stimulant is in the blood stream and is combated by the cells of organs most closely associated with the blood, *i.e.,* the spleen, liver and bone marrow, and in some cases general reactions may be regarded as local ones in strategically placed organs. The same types of cells are involved as in the local reaction, the polymorphonuclear leucocytes being mobilized first, with the macrophages playing the more important role. Endothelial cells, although in contact with blood-borne material, show very little phagocytic activity and the fibroblasts are rarely active.

The Cellular Response in Immunity. The above reactions, which are observed to occur in the non-immune animal, are markedly accentuated in the immune animal, and there is an increase in the number of macrophages frequently designated as a hyperplasia of the reticulo-endothelial system. The cooperative role of the humoral antibodies is of considerable significance; an antigen is localized by agglutination if cellular or by precipitation if in solution, and in either case the material is made more readily phagocytable by opsonization. Bacteria injected into the blood stream, for example, are rapidly removed in the immune animal by the fixed macrophages of the liver and spleen, and staphylococci injected into the skin are localized and phagocytosed in great numbers.[10] When antigen and antibody meet in the tissues of an immune animal, there are not only localization and opsonization of the antigen, but also a much heightened inflammatory reaction including a speeding up of the cellular response.

The Site of Formation of Antibodies. The question of the site of antibody formation is an old one and, since it now appears to be definitely established that antibody is modified serum globulin, it becomes one of the loci of globulin synthesis. Evidence has accumulated in support of the theory that antibody is formed in cells rather than from the humoral constituents of the blood, and that the cells responsible for the synthesis of immune globulin are those of the macrophage system, especially in the liver, spleen, lymph nodes and bone marrow. This evidence, generally indirect, is of a number of kinds. Thus, antibody formation is seriously interfered with by the removal of tissues containing large numbers of macrophages such as

[10] *Cf.* Cannon: Physiol. Rev., 1940, 20:89.
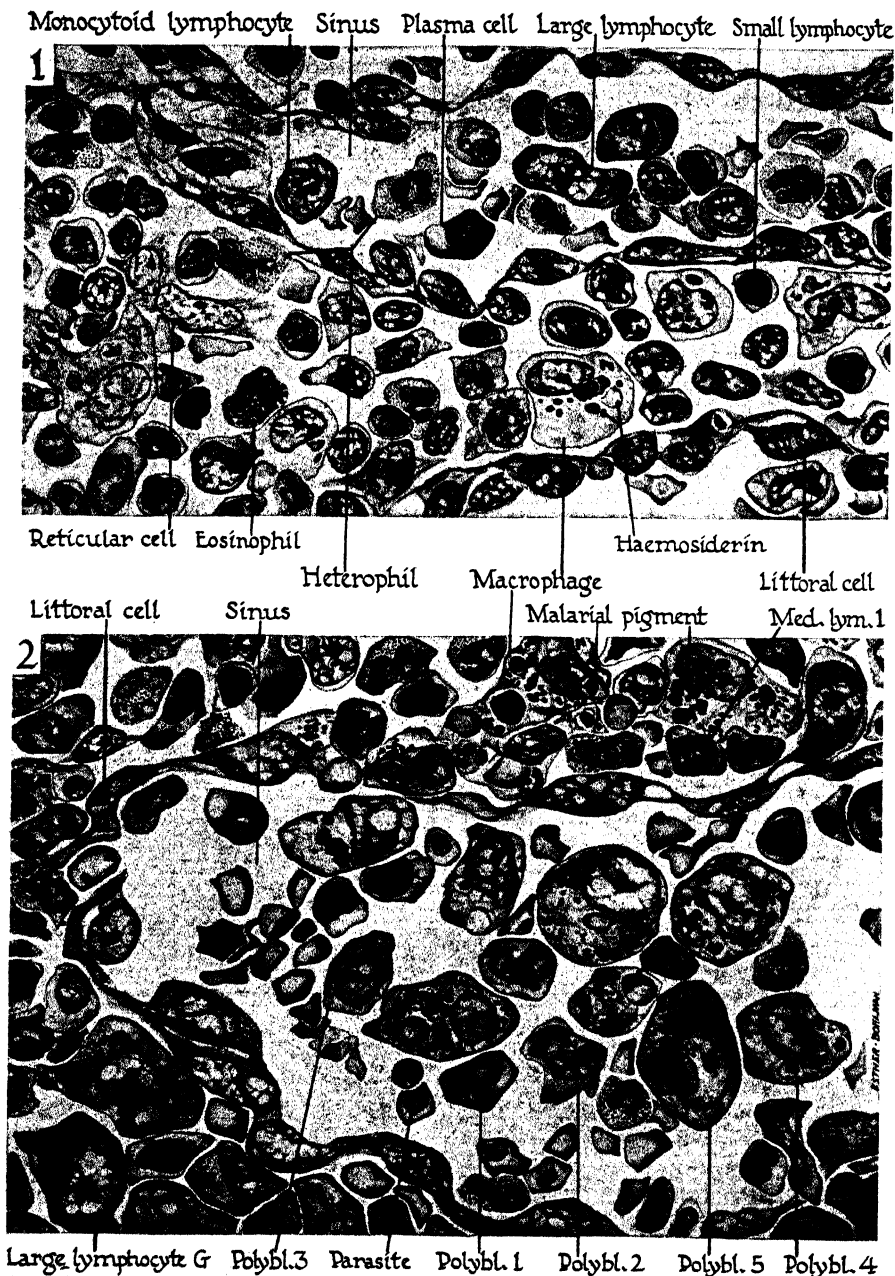
Fig. 39. Cells of the macrophage system. Sections of monkey spleen showing a portion of a venous sinus and a Billroth cord in the red pulp. *1*, Normal monkey; note the typical structure of the non-granular leucocytes, the reticular cells with indeterminate cytoplasm, and the rounded macrophages within the cord. *2*, Monkey killed ten days after infection with *Plasmodium cynomolgi*; note the large phagocytic cells absent from the normal spleen; the series of polyblasts labelled Polybl. 1–5 illustrate the progressive hypertrophy of the non-granular leucocytes into macrophages. Hematoxylin and eosinazure II; × 1240 (Taliaferro and Mulligan).

324
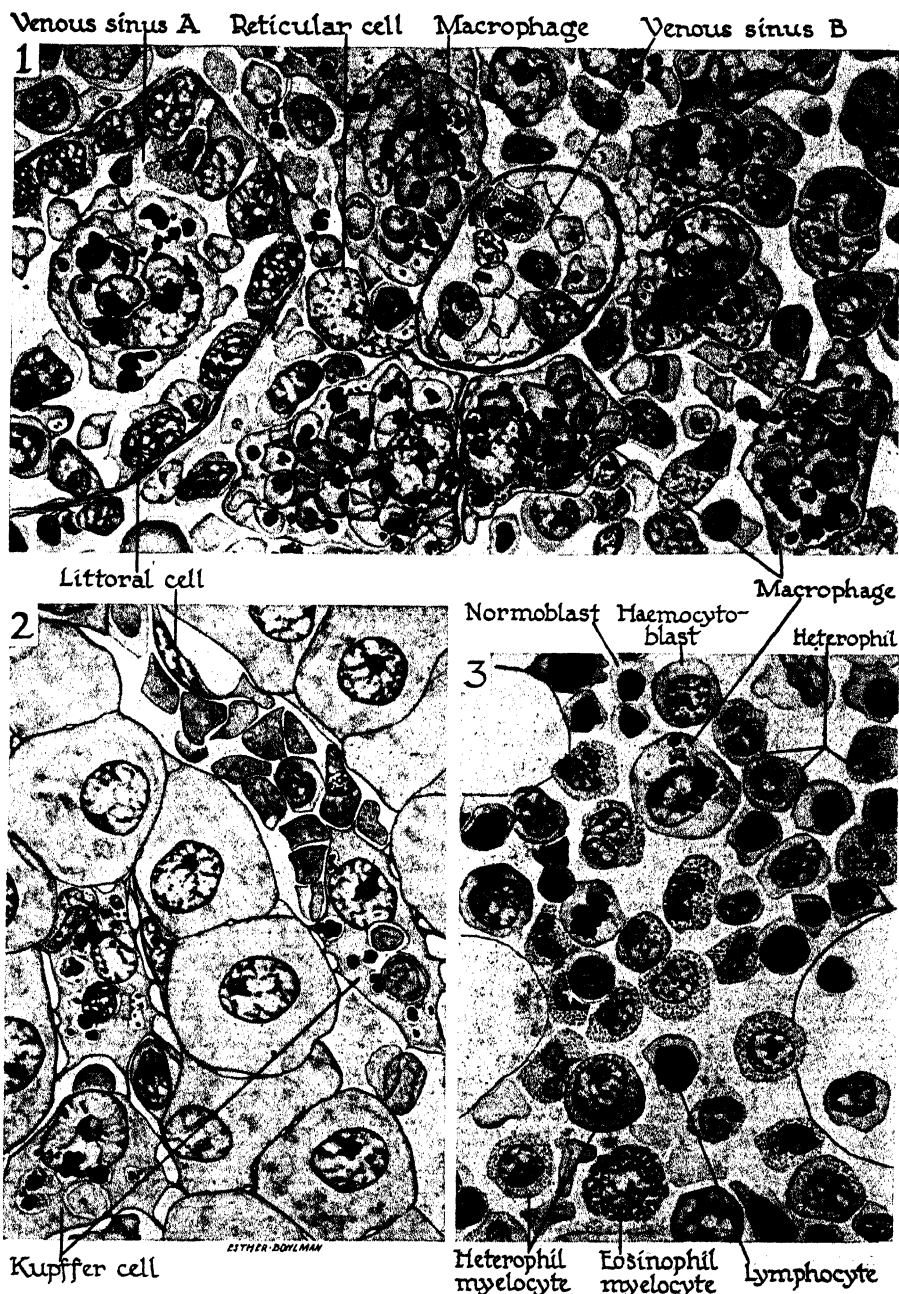
Fig. 40. Cells of the macrophage system. Sections of spleen, liver and bone marrow from a monkey killed fifteen days after infection with *Plasmodium cynomolgi*. *1*, A Billroth cord and two venous sinuses from the spleen; note the phagocytic activity of the macrophages and smaller polyblasts. 2, Section from the liver; the Kupffer cells are phagocytic but here less active than the spleen macrophages. 3, Section of bone marrow, showing a single, relatively small macrophage. Hematoxylin and eosin-azure II; × 1240 (Taliaferro and Mulligan).

the spleen. Similarly, blockade of the macrophage system by the injection of india ink (colloidal carbon) or other substances which are phagocytosed to such an extent that the phagocytic cells are fully occupied inhibits the immune response. Likewise, injury to hematopoietic tissue by irradiation, inoculation of benzene or nitrogen mustards also decreases the antibody response.

If it be true that these cells are responsible for the synthesis of antibody, it would appear to follow that they would form immune globulin in tissue culture. Carrel and Ingebrigtsen[11] found that the addition of goat red cells to cultures of guinea pig bone marrow and lymphoid tissue resulted in the appearance of hemolysin which, however, did not require complement for lysis. It has not been possible to repeat this work, *i.e.*, the addition of antigen to tissue culture of cells from normal animals, but many workers have observed the formation of antibody in cultures of tissue fragments, such as spleen and bone marrow, taken from animals that had been previously inoculated with antigen.

The inoculation of antigenic substances results in a marked lymphoid proliferation in the regional lymph nodes and in the spleen. In recent years observations have been reported by two groups of workers, Dougherty and his associates[12] and Ehrich and his associates,[13] which suggests a function of the lymphocyte in either the storage or formation of antibody. The evidence is of the following general nature[14]: Typhoid bacilli or sheep erythrocytes were injected into the plantar surface of the hind feet of rabbits; lymph was collected from the efferent lymph vessel of the popliteal lymph node, and the lymphocytes separated and washed by centrifugation; saline extracts were prepared by alternate freezing and thawing of the suspension of washed lymphocytes. Comparison of the antibody titer in the lymph plasma and lymphocyte extract showed that, five days after injection, the extract contained 8 to 16 times as much antibody as the plasma. In view of the long persistence of a solid immunity, this concept is surprising in that it relates antibody formation to a tissue as structurally and functionally labile as lymphatic tissue. This area is being actively investigated by a number of workers and in time no doubt the role and relative importance of the lymphocyte will become clear.

Local Immunity. The defense mechanisms functioning in the immune state have been assumed to be general ones and, for all practical purposes, equally effective throughout the body. It has been suggested, however, that these mechanisms are localized, or obviously accentuated, in certain tissues, not necessarily in tissues containing a large proportion of cells of the macrophage system, but tissues such as the skin, the intestinal mucosa, the nasal mucosa, etc.

[11] Carrel and Ingebrigtsen: Jour. Exp. Med., 1912, *15*:287.

[12] Dougherty *et al.*: Proc. Soc. Exp. Biol. Med., 1944, 57:295; *ibid.*, 1945, 58:135; *ibid.*, 1945, 59:172; Jour. Immunol., 1946, 52:101; review in Ann. New York Acad. Sci., 1946, 46:859, 882.

[13] Ehrich *et al.*: Science, 1945, *101*:28; Jour. Exp. Med., 1945, 81:73; *ibid.*, 1946, 83:373; *ibid.*, 1946, 84:157.

[14] Experiments reported by Harris, Grimm, Mertens and Ehrich: Jour. Exp. Med., 1945, 81:73.

Although attributable in some degree to the nature of the local defense factors, such as *p*H and the like, the predilection of an invading micro-organism for some particular part of the body such as the central nervous system might be regarded as indicative of a relative susceptibility of certain tissues and, conversely, a relative resistance on the part of others—in Ehrlich's terminology, the presence or absence of cell receptors. In a disease such as typhoid fever or erysipelas, for example, it should be necessary then that only the intestinal mucosa or the skin be immune, the other tissues being already resistant. This concept has been advocated particularly by Besredka.[15] The evidence upon which it has been based is generally regarded
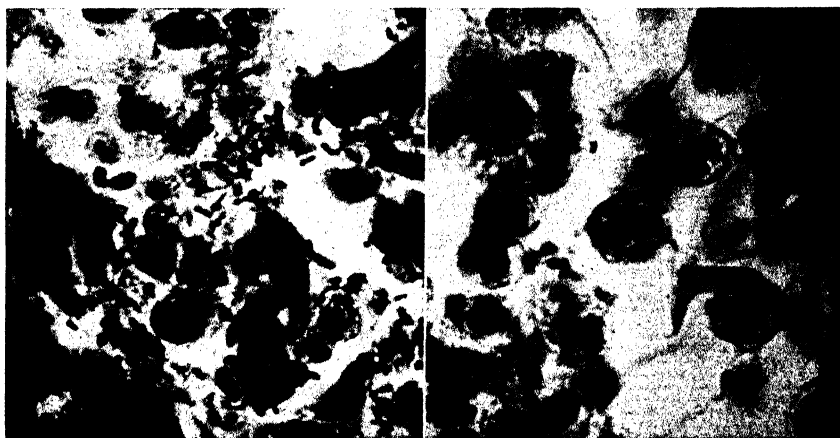
Fig. 41. The cellular response in the immune animal. Rabbits injected subcutaneously with virulent pneumococci. Left, normal animal showing a typical spreading lesion with many extracellular pneumococci and minimal phagocytosis by polymorphonuclear leucocytes. Right, immune animal, showing marked phagocytosis with intracellular agglutination and few extracellular bacteria. Sections stained by Gram's method; × 1050 (Cannon).

as relatively weak, and local immunity in Besredka's sense probably does not exist.

There is, however, good reason to think that local immunity, not of a particular tissue no matter where it may be in the body such as the skin, but of a local area, does exist. It would appear to follow that the presence of antigenic material in a particular area would stimulate the production of antibody by the cells of that area and, in consequence, higher anti-body titers would be found in the inoculated and immediately adjacent area than in the blood stream and other tissues. Such higher antibody titers have been demonstrated[16] in the nasal mucosa, for example, and it appears probable that, under circumstances in which antibody production takes place within a restricted area, that area may exhibit an accentuated immunity.

Natural Immunity. As pointed out elsewhere (p. 216), the defenses of the normal animal against infection are of two general types. The first,

15 Besredka: *Local Immunization.* Williams & Wilkins Company, Baltimore. 1927.

16 Cannon *et al.*: Proc. Soc. Exp. Biol. Med., 1931, 29:517, 675; Hartley: Jour. Inf. Dis., 1940, 66:44.

characterized by non-specificity with respect to the infectious agent, has been called resistance and discussed earlier. The second type, showing a greater or lesser degree of specificity, is considered here as natural immunity.

It is the rule rather than the exception that pathogenic microorganisms are sufficiently closely adapted parasites that they are able to establish infection only in more or less closely related species of hosts. Thus it is perhaps not to be expected that the same parasite will be able to infect organisms as widely different as higher plants and mammals. It is of particular interest, therefore, that *Pseudomonas pyocyanea*, a not uncommon pathogen of man and higher animals, has been found able to infect tobacco plants and may be identical with the plant pathogen *Phytomonas polycolor*.[17]

While in Ehrlich's terminology it might be said that the cellular organization of the plant lacks receptors for the human pathogen, there is evidence of a positive type of resistance to such infection. Antibiotic substances, similar to those produced by bacteria and fungi (p. 128), have been found in a number of higher plants. Thus, extracts of cabbage, turnip, onion and barberry plants inhibit the growth of *Bacterium coli* and *Bacillus subtilis*,[18] chlorophyll and related compounds have been found to inhibit the growth of the tubercle bacillus,[19] and substances which inhibit the growth of *Staphylococcus aureus* and *Bacterium coli* have been found in a large number of angiosperms.[20] None of the plants, however, is known to produce anything resembling antibodies.

Natural immunity is closely associated with the specific immune response, an immunity which is expressed in the form of the bactericidal and opsonic powers of normal blood, in the presence of the so-called normal agglutinins and the like. Such antibodies are generally present only in low titer, and the immunity associated with their presence in man is of a low grade but is possibly a significant part of that complex designated "resistance" in an earlier chapter. In many instances antibodies may be found under circumstances in which it is highly improbable that the animal has come in contact with the infectious agent; it has been noted, for example, that cattle sera will not infrequently neutralize the yellow fever virus and, more often, contain agglutinins for bacteria such as the typhoid and dysentery bacilli, the cholera vibrio, and certain of the rare varieties of Salmonella. Although present in low titer, such normal agglutinins appear to be no different from the immune agglutinins in adsorbability, heat resistance and the like. The normal macrophage response does not involve this specific element, for all kinds of intact particles are phagocytosed. The question of the origin of these antibodies is, then, of some significance and it may be asked whether or not specific antibodies may be formed in the absence of antigenic stimulation.

[17] Elrod and Braun: Jour. Bact., 1942, 44:633.

[18] *Cf*. Sherman and Hodge: Jour. Bact., 1936, *31*:96; Dick: Arch. Surg., 1940, *41*:287; Fuller and Higgins: Food Research, 1940, *5*:503.

[19] Daly, Heller and Schneider: Proc. Soc. Exp. Biol. Med., 1939, 42:74.

[20] Osborn: Brit. Jour. Exp. Path., 1943, 24:227; see also Pratt *et al.*; Science, 1944, 99:351; Tsuchiya *et al.*: Jour. Bact., 1944, 47:422; Lucas and Lewis: Science, 1944, *100*:597.

Natural Antibodies. That the formation of some antibodies is genetically determined and takes place in the absence of antigenic stimulus is established in the case of the antibodies which determine the human blood groups. It is not unreasonable to suppose that in other instances the arrangement of polar forces on the surface of the normal globulin molecule might be such as to make possible to a greater or lesser extent the specific adsorption of antigenic substances. The probability that this will occur is, however, not great, and it is unlikely that such an explanation is generally valid.

Inapparent Infection. A second possibility that accounts for the presence of normal antibodies is that of inapparent infection. A case in point is that of the appearance of diphtheria antitoxin in the blood in the absence of clinically recognizable infection; it is well known that the proportion of Schick-negative individuals increases in succeeding age groups. It is definitely established that the indicated formation of antitoxin is an immunity resulting from inapparent infection, *i.e.*, the carrying of virulent diphtheria bacilli in the throat, and the antigenic stimulus of the presence of small amounts of diphtheria toxin (p. 621). The agglutinins for Flexner dysentery bacilli and some of the common species of Salmonella associated with food poisoning not infrequently present in normal human sera to titers of 1:80 to 1:160 in all probability are a consequence of subclinical infections. It is probable that such a sequence of events occurs in a number of diseases and that so-called normal antibodies, such as the neutralizing power of normal adult serum for poliomyelitis virus, measles virus and other infectious agents, are, in fact, immune antibodies. The manner in which a pseudoracial immunity may be produced through infection has been discussed elsewhere (p. 219).

Common Antigens. As indicated above, however, there are many instances in which it is extremely unlikely that the animal whose serum agglutinates a given bacterium came in contact with it either as a mild infection or otherwise. It is difficult to conceive, for example, of the ordinary cow in the United States coming in contact with the cholera vibrio. In such cases it is not necessary to assume either that the observed antibodies arose *de novo* through some genetic or maturation mechanism or that there was contact with the particular microorganism. It is only necessary to assume that the animal has come in contact with an immunologically similar antigen. The likelihood that this will occur is clearly a function of the frequency with which immunological relationship or identity is shared by diverse organisms and the probability of contact with the unrelated form.

In addition to heterophile antigen, whose apparent random occurrence has been discussed elsewhere, immunologically similar antigens have been found to occur in a variety of seemingly unrelated organisms, and it is probable that many more have not as yet been discovered. For example, cross-reactions occur between Type 2 pneumococcus polysaccharide and Type B Friedländer's bacillus, certain species of yeasts, gum acacia and other vegetable gums, and some strains of *Bacterium coli* and *Bacterium aerogenes;* the polysaccharide of Type 14 pneumococcus is immunologically similar to the specific antigen of human blood group A; a constit-

uent present in peptone is immunologically related to certain streptococcus antigens (group C); the capsule of the anthrax bacillus appears to be immunologically identical with that of *Bacillus mesentericus;* the plague bacillus and paratyphoid B are immunologically related. From these examples[21] it will be clear that immunologically similar antigens may be distributed in an apparently random fashion in unrelated organisms and it is probable that in some, if not many, instances in which antibodies may be demonstrated for some microorganism with which infection or contact is unlikely, the antibodies are immune rather than "normal," and a consequence of exposure to a similar antigen.

In keeping with this discussion it may be pointed out that there is a strong possibility that there is no such thing as a normal specific antibacterial immunity and that immunity in which a specific antibody may be demonstrated is one that arises as a consequence of exposure to the antigen. The incubation period following injection, for example, provides the opportunity for an immunological response that is difficult to rule out in many instances. In this connection it is of interest that attempts to establish a monoflora of *Bacillus subtilis,* an organism generally regarded as a harmless saprophyte, in bacteriologically sterile rats have resulted in a number of deaths from *B. subtilis* septicemia.

Acquired Immunity. Specific immunity against infection may be of two types. The one, *active immunity,* is due to the direct participation of the host and is gained at the expense of the organism acquiring it. This active immune state, manifested by the presence of antibodies, a heightened cellular reactivity, and a marked increase in resistance to the infection, arises as a consequence of the stimulus of antigen present in the tissues. The antigen may be inadvertently present, as in an attack of disease, or it may be purposely introduced into the tissues in such a form that, while its antigenic qualities are little if at all impaired, disease is not produced. Active immunity may, then, be artificially produced by the parenteral inoculation[22] of appropriate antigenic material. In general terms, the methods by which active immunization may be accomplished are:

(1) The inoculation of living, fully virulent bacteria
    (a) rarely by a route favorable to infection but
    (b) occasionally by a route unfavorable to infection or
    (c) in conjunction with protective antiserum;
(2) the inoculation of attenuated bacteria of greatly reduced virulence for the host;
(3) the inoculation of bacteria killed by
    (a) heat or
    (b) antiseptics;
(4) the inoculation of bacterial products
    (a) secreted during life or
    (b) liberated by the autolysis of dead cells;
(5) the inoculation of bacteria unrelated to the production of the specific infection.

These may be briefly illustrated.

(1) Immunity produced by the introduction of living, virulent bacteria

[21] The earlier literature is reviewed by Ingalls: Jour. Immunol., 1937, 33:123.

[22] Oral administration of antigenic material is efficacious only in so far as unmodified antigen is absorbed through the intestinal wall and, compared with parenteral inoculation, is an uncertain and inefficient method.

is practically identical with the immunity that results from an attack of disease after natural exposure. In experimental work the varying facility with which this mode of immunization can be effected is in part dependent upon the susceptibility of the organism to the particular parasite, and a highly susceptible animal can be immunized in this way only with great difficulty or not at all. The successful use of living cultures involves the administration of small non-fatal doses which are increased as immunity develops.

The relative insusceptibility to infection by some particular route may be taken advantage of, as in Ferran's method, now superseded, for protective vaccination against Asiatic cholera; natural infection occurs via the alimentary tract and subcutaneous injection of the virulent vibrios is followed by a local inflammation but not by a general infection with serious consequences.

The simultaneous administration of virulent microorganisms and protective antiserum provides preformed antibody for combating the invader while allowing the immune reaction of the host to develop. This method is seldom used for the immunization of human beings but is common in some other instances; hogs may be immunized against hog cholera, for example, by simultaneous injection of virus-containing blood and antiserum.

(2) As indicated elsewhere, the virulence of a bacterial culture may be greatly diminished in a variety of ways, such as cultivation under unfavorable environmental conditions and by passage through animal species other than that of the host in question. Perhaps the most familiar examples of the use of such attenuated material are those of smallpox vaccination and inoculation against rabies. In the first instance the passage of smallpox virus in the cow greatly reduces its virulence for man but inoculation with the altered virus of cowpox protects against smallpox infection, and in the second rabies virus from dogs is "fixed," in Pasteur's terminology, or attenuated by serial passage in rabbits. This method is particularly useful in immunization against the filterable virus diseases, since cultures are not available and "killing" destroys the antigenic properties of these agents. (See, however, p. 853.)

(3) Suspensions in physiological salt solution of bacteria killed by heating to 55° to 60° C. for thirty minutes or by treatment with formaldehyde or phenol are widely and successfuly used in man against typhoid and paratyphoid fevers and against a variety of microorganisms in the laboratory. Such vaccines are, in general, most useful in infections in which the microorganism does not produce a soluble toxin but contains an endotoxin. This method has the obvious advantage of avoiding all danger of infection while at the same time introducing into the body the substances most intimately connected with the bacterial cell and its activities.

(4) The use of products of the bacterial cell for immunization purposes finds widest application in the case of those organisms which produce soluble toxins. As pointed out above, a solid immunity against diphtheria may be secured by stimulating the production of antitoxin. It is possible to build up such an immunity by using extremely small amounts of unmodified toxin in the early injections with gradual increases as immunity develops. In general, however, it is much more satisfactory to use neutralized or

detoxified toxin. In the first instance a neutral mixture of diphtheria toxin and antitoxin (TAT) is frequently used with satisfactory results; the complex breaks down slowly in the body, liberating free toxin which acts as the antigen. Toxoid prepared by treatment with formalin which has lost its toxicity but retained its antigenicity is generally used at the present time in a partially purified form precipitated by alum. The alum-precipitated toxoid is of some advantage since the toxin-alum complex breaks down slowly in the tissues, liberating toxoid slowly to provide a prolonged antigenic stimulus.

It is difficult to distinguish between bacterial products secreted during life and the products of autolysis; it has been pointed out previously, for example, that usually the soluble toxin titer of a culture rises, not coincident with the phase of active growth, but after cell multiplication slows down and breakdown of the formed cells begins. Studies have been made, however, on the antigenic qualities of autolyzed cultures and of cell extracts, but such antigens are not of great practical value since they are contained, for the most part, in suspensions of killed cells.

(5) Some degree of immunity toward specific infections may be developed by the use of certain kinds of bacteria or bacterial products entirely foreign to the infection in question. In this category, for example, is the undoubted protection conferred against anthrax by the injection of *Bacterium prodigiosum* or *Ps. pyocyanea* or their products. A number of instances of this sort have been described in connection with studies on common antigens and need not be considered further here.

Irrespective of the antigen employed, the animal body requires a period of time to respond with antibody production and for a high degree of immunity successive antigenic stimuli are required. Early injections may consist of killed bacteria, followed by the inoculation of living, attenuated or virulent cells. The process of forcing a very high degree of immunity upon an experimental animal is often termed *hyperimmunization*. If the antigen is injected in such a form that it is slowly absorbed, thus providing a prolonged stimulus, repeated injections may not be necessary. A single injection of alum-precipitated diphtheria toxoid, for example, may give about 80 per cent Schick negatives. Attempts have been made to use *lipovaccines*, suspensions of bacteria in oil, to promote such low absorption, but these are generally unsatisfactory because they are difficult to sterilize.[23]

Antibody is presumably first formed in the antibody-producing cells and then spills over into the body fluids and blood stream. It is probable that the lag between inoculation and the appearance of serum antibody is in part due to a mobilization of the processes of synthesis of immune globulin, and in part to the time required for the accumulation of antibody shed by the cells to detectable concentration in the body fluids. Antibody also appears in some of the secretions and excretions. The virus-inactivating agent in the nasal mucus (p. 226) is regarded by some as antibody, and antibody has been found to occur in the feces and urine of immunized animals and man.[24]

[23] See, however, Halbert, Mudd and Smolens: Jour. Immunol., 1946, 53:291.
[24] Burrows, Elliott and Havens: Jour. Inf. Dis., 1947, 81:261; Harrison and Banvard: Science, 1947, 106:188; Burrows and Havens: Jour. Inf. Dis., 1948, 82:231.

The former is of some interest in that it may be associated with immunity to the enteric infections in which the infection is confined to the lumen of the bowel and superficial layers of the intestinal mucosa.

An animal may be simultaneously immunized against more than one antigen with no significant effect on the immune response. Typhoid vaccine, for example, commonly contains typhoid, paratyphoid A and paratyphoid B bacilli (TAB), and other so-called polyvalent vaccines containing a variety of microorganisms are effective immunizing agents.[25]

The immunity produced is variable to some degree and dependent upon the efficacy of the antigen used. Some kinds of bacteria are "good antigens," such as the typhoid bacillus, while others, such as the gonococcus, are "poor antigens" and no effective immunity may be produced. The reason for this is not known. When an active immunity is produced, however, it is generally of long duration and effective over a period of years.

The immune response, as indicated by antibody titer, generally is apparent by the second of a series of injections and the titer increases with succeeding injections until an upper limit is reached. With cessation of inoculations the antibody titer slowly declines and within a few weeks has reached a very low level. Subsequent injection brings about an immediate antibody response, much more rapid and pronounced than that of the initial immunization. It is of some interest that this secondary response may be brought about, although to a lesser degree, by the injection of a heterologous antigen, a phenomenon that has been termed the *anamnestic reaction:* this stimulation to fresh production of one antibody when a new antigen is injected has been an important obstacle to theories of antibody production such as retention of antigen in the cells, etc. It may be noted here that the injection of any antigenic substance stimulates the mobilization of the defense mechanisms of the body, a phenomenon which is made use of in *non-specific protein therapy;* the injection of sterile milk, for example, may markedly stimulate the body to combat invading microorganisms. It will be clear that, under the circumstances, any interpretation of the beneficial effect of vaccine therapy of infectious diseases must be made with caution.

Passive Immunity. In contrast to active immunity, passive immunity involves no active generation of protective substances by the immunized animal. The latter is simply the recipient of antibodies formed in the body of another animal and transferred to the individual to be protected. Such passive transfer occurs in nature from mother to offspring, *in utero* via the placental circulation in man, apes, and rodents in which there is only one layer of cells between the maternal and fetal circulatory systems, and after birth via the colostrum in ruminants in which there are four layers of cells between the two circulatory systems.[26] The direct absorption of immunologically unaltered antibody from the lumen of the bowel in the adult guinea pig has been observed also.[27]

Artificial passive immunization is brought about by the injection of

[25] For recommended combinations see Amer. Jour. Pub. Health, 1944, 34:452.
[26] See the review in the Lancet, 1941, *i*:44.
[27] Burrows and Havens: Jour. Inf. Dis., 1948, 82:231.

immune sera such as the antitoxic sera of horses immunized against diphtheria toxin, tetanus toxin and similar antigens, or of convalescent sera taken from recovered human cases of the disease in question. Passive immunization is most effective with antitoxic sera; with exceptions such as virus neutralizing sera, antipneumococcus sera and the like, antibacterial sera are generally not highly effective either prophylactically or therapeutically.

Unlike active immunity, passive immunity is not of long duration, generally not more than two or three weeks. Repeated injections of tetanus antitoxin, for example, must be made at weekly intervals as long as the danger of infection remains. Horse serum globulin acts as a foreign protein in the human body and tends to be eliminated as such. Passive immunization with homologous serum is possibly of somewhat longer duration.

HYPERSENSITIVITY

The response of the animal body to the presence of antigen in the tissues is clearly an advantage when the antigen is a toxin or the cell substance of a pathogenic microorganism. It was early apparent, however, that an initial inoculation of an antigen may so sensitize the animal that subsequent inoculation results in the development of a typical symptom complex and perhaps death, and in such cases the animal is said to be hypersensitive. This effect is apart from such phenomena as the neutralization of toxin by antitoxin and is most striking when the antigen is initially bland. While it may play some part in effective immunity, there is also reason to believe that hypersensitivity to the cell substance of bacteria may, in some instances, contribute to the pathology of the disease resulting from infection as, for instance, in acute rheumatism and arthritis, and hypersensitivity to non-living antigens may also result in disease of non-infectious nature. Hypersensitivity is quite general and may take a variety of forms in both experimental animals and man.

Anaphylaxis.[28] The sensitizing effect of an initial inoculation of antigen was observed by Richet in 1902 in a study of the immunization of dogs with toxic extracts of the sea anemone. About this same time Theobald Smith noted the lethal effects of second and third inoculations of diphtheria toxin-antitoxin in the guinea pig when the inoculations were widely spaced, and in 1905 Otto found that the active agent in the mixture was horse serum. This was subsequently studied in considerable detail by Rosenau and Anderson and the general picture is now quite clear.

If a guinea pig is inoculated with horse serum at intervals of perhaps four to eight days, it responds with the development of an immunity and precipitating antibody appears in the serum. If, however, an interval of ten to twelve days elapses between the first and second inoculations, the animal becomes hypersensitive as a consequence of the first inoculation and the second is highly toxic. The state of hypersensitivity so induced by the *sensitizing dose* is designated *anaphylaxis* and the reaction produced by the second, or *shocking dose*, is *anaphylactic shock*. This phenomenon of an-

[28] Dragstedt: Physiol. Rev., 1941, 21:563.

aphylaxis is a laboratory artifact in that it results from the purposeful inoculation of antigen into the tissues, and shock is produced by the rapid inoculation of relatively large amounts of antigen, usually directly into the blood stream. It is artificial in that it occurs only under conditions set up by man, in experimental inoculation of animals and in serum therapy in man.

The sensitizing dose may be exceedingly small, as little as 0.000001 ml. of horse serum in the guinea pig, and varies with different antigens and different experimental animals. The shocking dose is considerably larger, 0.1 to 0.01 ml. of horse serum for the guinea pig. Within a certain range the severity of the shock produced is directly related to the size of the shocking dose, *i.e.,* mild shock is produced by a small dose and lethal shock by a larger dose. The route of inoculation of the sensitizing dose is not important. For some animals and some antigens several sensitizing doses may be required to develop hypersensitivity, but in any case the doses must be small and widely spaced. A single dose suffices to shock and the route of inoculation is important. Intravenous and intracardial inoculation is most effective, and in the guinea pig, which is the most readily sensitized and shocked of the usual experimental animals, intraperitoneal inoculation of the shocking dose is effective but the reaction is delayed and larger doses are required. Once established, the anaphylactic state persists more or less indefinitely but diminishes appreciably in the guinea pig after a few weeks. If the shock produced is not lethal, the animal is temporarily refractory, that is to say, is *desensitized,* but hypersensitivity reappears, in the case of the guinea pig after perhaps two weeks or less. Guinea pigs, rabbits and dogs are the common experimental animals, but birds may be sensitized, while mice, rats and monkeys can be sensitized only with difficulty and then irregularly. The symptoms and postmortem pathology are essentially identical within a given animal species regardless of the antigen used, but differ from one animal species to another.

The Guinea Pig. Following inoculation of the shocking dose the animal remains quiet for a few moments but very soon becomes restless, the fur becomes ruffled, and the animal rubs its nose and sneezes. Clonic and tonic convulsions set in, the animal falls to its side, respiration becomes labored and the animal dies gasping for breath while the heart continues to beat. This symptom complex is produced in varying degree in non-fatal shock. The outstanding postmortem findings are the marked distention of the lungs and hemorrhages on the under side of the diaphragm. The lungs remain distended after the thoracic cavity is opened and when removed from the body and cut. The bronchioles are sharply constricted with the retention of air in the alveoli, and the immediate cause of death is suffocation. The contraction of the bronchioles is not prevented by vagotomy or curare and is muscular or immediately neuromuscular in origin.

The Rabbit. The rabbit is less susceptible to anaphylactic shock than the guinea pig but fatal shock may be produced by intravenous inoculation of relatively large amounts of antigen. The symptoms and pathology differ sharply from those in the guinea pig with absence of difficulty in respiration. The animal falls on its side, shows convulsive movements and passage of

feces and urine. Respiratory movements may continue briefly after the heart has ceased to beat. On autopsy the pulmonary dilatation found in the guinea pig is absent and the most conspicuous and characteristic feature is the extreme dilatation of the right side of the heart. The immediate cause of death is heart failure resulting from constriction of the branches of the pulmonary artery and consequent dilatation.

The Dog. Anaphylactic shock in the dog occurs in two stages. The first is characterized by restlessness, the animal vomits and passes urine and feces, and collapses with signs of extreme muscular weakness and respiration is labored. The blood pressure falls rapidly as a consequence of capillary dilatation and stasis in the tissues. In non-fatal shock recovery from this stage is rapid, but in fatal shock the weakness is progressive, vomiting and diarrhea continue, convulsions may occur and the animal becomes comatose and dies. On autopsy the outstanding feature is the congestion of the viscera and the pronounced distention and congestion of the liver, which is a consequence of constriction of the hepatic veins and injury to the liver cells resulting in edema from the transudation of fluid.

In addition to the characteristic phenomena noted above, anaphylactic shock also has common features which may be more or less pronounced in different animal species. Fall in body temperature is usual and leucopenia occurs which results from aggregation of leucocytes in the capillaries. Coagulation time of the blood is increased, usually most marked in the dog, as a result of the liberation of heparin rather than direct interference with the clotting mechanism, and serum complement is reduced. Congestion and hemorrhage of the gastro-intestinal tract are common postmortem features.

Local Anaphylaxis. Local occurrence of anaphylaxis was observed by Arthus in 1903 and is called the *Arthus phenomenon.* If rabbits are injected repeatedly with antigen such as horse serum, a local reaction appears which becomes more and more intense as the inoculations are repeated. The site of inoculation first shows a transient swelling, but later the swelling and edema persist and progress to induration and localized necrosis. The local phenomenon is a result of a general sensitization since later inoculations need not be in the same site as earlier ones. The Arthus phenomenon appears to be confined to the rabbit among experimental animals, or at least is very difficult to produce in other animals, but occurs with some frequency in man, as in protracted serum therapy with successive intramuscular inoculations of antiserum, or in prolonged series of inoculations as in the earlier Pasteur treatment for rabies.

Passive Anaphylaxis. Anaphylactic hypersensitivity is passively transferable from sensitized female guinea pigs to offspring, and by inoculation with serum from either sensitized or immunized animals. It can be produced with a high degree of consistency by inoculation of guinea pigs with immune rabbit serum, and in general the sensitizing effect is proportional to the precipitin content of the antiserum. The recipient is not immediately sensitized following the injection of antiserum and usually, though not invariably, a period of some hours elapses before sensitivity appears. If antigen is injected first, followed by the inoculation of antiserum, *reversed passive anaphylaxis* occurs, also usually some hours after the inoculation of serum.

Function of Haptenes in Anaphylaxis. The specificity of the anaphylactic reaction may be, and probably frequently is, determined by haptenes. In general sensitization can be accomplished only by complete antigen but in a few instances simple compounds, including arsphenamine, picryl chloride and 2, 4-dinitrochlorobenzene, have been shown to sensitize guinea pigs and sensitization to arsphenamine has been observed in man. Haptenes will usually not elicit shock, though some of the larger molecules such as azo dyes will do so, but the inoculation of haptene specifically prevents shock by inoculation of the complete antigen. The role of haptenes in specificity is further shown by the use of conjugated antigens containing the same haptene but different proteins; thus, guinea pigs sensitized with a conjugated globulin can be shocked with the corresponding conjugated albumin.

Anaphylactic Shock in Isolated Tissue. It will be clear from the foregoing discussion that the occurrence common to anaphylactic shock in different animals is a contraction of the smooth muscle, predominantly in the bronchioles in the guinea pig, in the pulmonary artery in the rabbit, and in the hepatic veins in the dog, and in all three the gastro-intestinal tract is affected. It is, then, of considerable interest that anaphylactic shock, manifested as a sharp and abrupt contraction, can occur in isolated smooth muscle. This was observed by Schultz in 1910 using portions of intestine, and studied in detail by Dale with uterine horn, and is now generally known as the *Schultz-Dale technique.* The uterine strip is suspended in a bath of Ringer's solution with one end tied fast and the other attached to a kymograph, and the contractions are recorded when antigen is added to the bath. The reaction is exquisitely sensitive, a sensitized strip giving typical contraction in final dilutions of horse serum antigen as high as 1:1000 million, and sharply specific. All of the essential features of anaphylaxis may be reproduced *in vitro* by this method, including active or passive sensitization of the uterine strip *in vivo*, passive sensitization *in vitro*, specific desensitization, etc. It is significant too that uterine horn from immunized as well as sensitized guinea pigs will respond to antigen with shock.

Mechanism of Anaphylactic Shock. It will be clear that anaphylaxis rests on an immunological basis and that shock is the result of an antigen-antibody reaction. There is good reason to believe that the union is one of antigen with intracellular or sessile antibody. For instance, as just indicated, sensitization and shock may be produced in isolated tissue, and the incubation period in passive sensitization is regarded as necessary for the taking up of inoculated antibody by the cells. Desensitization consists of saturating, or nearly saturating, sessile antibody, and the protective effect of immunity is a prevention of antigen reaching intracellular antibody by union with serum antibody. While by no means final, these interpretations are generally accepted. The means by which shock is produced through antigen-antibody union is not so clear and two general theories have been proposed.

One of these is the *anaphylatoxin theory* which is based on the occurrence of *anaphylactoid reactions* produced by the inoculation of normal serum made toxic by treatment with kaolin, starch and other similar adsorbing agents. On inoculation such toxified sera give a reaction which is closely similar to, but not identical with, anaphylactic shock. Furthermore, peptone

shock, produced by the inoculation of peptone, is also very similar. The theory, originally based on the assumption that the causative antigen-antibody reaction occurs in the blood stream but not completely inconsistent with an intracellular union, postulates the neutralization of an inhibitory agent such as antitrypsin by the colloidal antigen-antibody complex, and proteolytic digestion is thought to occur with the formation of the so-called anaphylatoxin which is directly responsible for the smooth muscle contraction. While this kind of explanation remains a possibility, it is not generally accepted.

The other theory, which has gained wide acceptance, rests on the assumption that the intracellular union of antigen and antibody results in the liberation of histamine. There is a good deal of indirect evidence in support of the role of histamine in anaphylactic shock. For example, the reaction produced by the inoculation of histamine is indistinguishable from anaphylactic shock. There is no reason to believe that antibody is fixed primarily or even predominantly in smooth muscle cells, but there is a high correlation between histamine sensitivity and the cells concerned in anaphylactic shock. Furthermore, the tissues affected in anaphylactic shock are those that show the highest content of histamine; there is some evidence which suggests that leucocytes are a source of histamine and, as indicated above, they aggregate in affected tissues in shock. In addition many, though not all, substances showing antihistamine activity appreciably or markedly modify anaphylactic shock; of these epinephrine and ephedrine are among the most effective. On the whole, it seems probable that histamine plays an important part in anaphylactic shock, and there is some evidence that choline or acetylcholine may have some minor significance. However, the basic question, in what manner histamine is liberated as a consequence of the union of antigen with sessile antibody, still remains unanswered.

Serum Sickness. The inoculation of man with antiserum, usually from the horse, produces in some persons a characteristic syndrome termed serum sickness. The symptoms include rash, often urticarial in nature, fever, joint pains, some edema, and swelling of the lymph glands regional to the site of inoculation, in combinations and emphasis that are variable from one individual to another. This reaction is to be distinguished from the febrile and local reactions commonly following the inoculation of foreign protein and is most commonly attributable to a hypersensitivity to horse serum irrespective of its antibody content, though essentially similar reactions may be produced following the inoculation of toxoids, vaccines, etc., as a consequence of hypersensitivity to constituent antigen. The incubation period may be as short as two hours or as long as twenty-four days, and most often is eight to twelve days. The reaction may follow initial inoculation of horse serum, but more frequently there is a history of prior inoculation. It is specifically antagonized by epinephrine, ephedrine, etc., and, like anaphylactic shock, is presumably a consequence of the sudden liberation of histamine in toxic amounts.

The hypersensitive individual gives an immediate reaction, *i.e.*, appearing within ten to twenty minutes, to the intradermal inoculation of 0.1 ml. of 1 : 10 dilution of horse serum (a similar test for sensitivity to diphtheria

toxoid is the Moloney test, p. 618), or the instillation of a drop of horse serum in the conjunctival sac. In the first instance an irregular wheal surrounded by an erythematous zone constitutes a positive reaction, and in the second a diffuse conjunctivitis appears in the hypersensitive individual. Desensitization may be effected by the inoculation of very small amounts of serum, usually doubled in successive inoculations, over a period of time; the inoculations may be given at intervals of fifteen to twenty minutes because of the rapidity with which the reaction occurs.

The question may be raised as to why serum sickness rather than anaphylactic shock occurs in the hypersensitive individual. In general there seems to be little tendency in man to generalized shock in this and other manifestations of hypersensitivity and, as in dogs and monkeys, there is a tendency to localization of the manifestations of the reaction to the respiratory tract, the gastro-intestinal tract and the skin. A number of instances of typical anaphylactic shock have been reported in man though it is rare, and when generalized shock occurs it is often fatal.

Allergy. Serum sickness is perhaps best regarded as a form of anaphylaxis in man, but only a small part of the manifestations of hypersensitivity are those of serum sickness, and the remainder may be grouped under the general head of allergy. Like anaphylactic shock, an allergic reaction is a consequence of the union of antigen with sessile antibody. The antigen is often designated *allergen* and the corresponding antibody *reagin* because it was thought earlier that they differed from antigen and antibody. It is now generally recognized that there are no essential differences but the terms persist even though a number of workers have urged that they be dropped.

There are a number of differences between anaphylaxis and allergy which are rather of degree than of kind, but which in the aggregate tend to distinguish them. Thus, allergy is naturally acquired while anaphylaxis is artificially produced, allergy is often a hypersensitivity to non-protein antigens while anaphylaxis is only rarely so, allergic hypersensitivity is of long duration while that of anaphylaxis is limited, desensitization is usually difficult and incomplete in allergy and effective in anaphylaxis, and edema is a prominent feature of the allergic reaction and smooth muscle contraction a minor factor while the reverse is true in anaphylaxis.

Heredity. It has been a matter of some interest that inheritance of a predisposition to at least some forms of allergy is an important, perhaps often a determining, factor. The constitutional factor is a predisposition only and contact with the antigen is essential to the development of the allergic state. Inheritance in man is frequently difficult to demonstrate, but detailed studies on familial association have made it highly probable that predisposition to allergic hypersensitivity is genetically determined. It has been suggested[29] that the predisposition is determined by a pair of allelomorphic genes, H determining non-allergy and h allergy. The possible genotypes are HH or pure normal, hh determining allergy which develops before puberty, and Hh the normal transmitter in which allergy may develop after puberty. In confirmation of observations on man, the inheritance of predisposition to

[29] Wiener, Zieve and Fries: Ann. Eugenics, 1936, 7:141.

sensitization has also been shown in experimental animals.[30] The significance of heredity in allergy has been said to distinguish this form of hypersensitivity from anaphylaxis but it is not clear that this is a basis of distinction.

Forms of Allergy. The allergic state is manifested in a variety of forms which are determined by two interrelated factors, the portal of entry of the antigen and the tissue predominantly affected, usually referred to as the *shock organ.* Thus the antigen may be inhaled, ingested, injected or may simply make contact with the skin. The tissues affected are those of the upper respiratory tract, the gastro-intestinal tract and the skin. These combinations give rise to a number of commonly occurring, well-defined clinical types, *viz.:*

Hay Fever. This is a seasonal allergy produced by the inhalation of pollens from trees, grasses and weeds, and time of occurrence is determined by time of pollination, *i.e.,* trees and grasses from late spring to midsummer and weeds in late summer and early fall. In the United States ragweed pollen is one of the most common offenders. Non-seasonal hay fever is a result of hypersensitivity to animal danders, orris root (a constituent of many cosmetics) and house dust. The mucous membranes of the upper respiratory tract are affected primarily.

Asthma. Essentially the same inhaled antigens are responsible for asthma as for hay fever and in addition book bindings, straw and similar materials are sometimes involved. The individual may become sensitive to bacteria of the normal flora of the upper respiratory tract, giving rise to so-called endogenous asthma. The shock organ is the lining and musculature of the bronchi in bronchial asthma, by far the most common type, and swelling of the mucosal lining and spasm of the muscles results in obstruction of the smaller bronchioles and consequent difficulty in breathing. Allergic asthma may also result from the ingestion of foods, such as eggs, milk, wheat, or various drugs. It may be noted that asthma is a symptom complex and not always allergic in etiology.

Dermatitis. Dermatitis of allergic etiology may result from contact with the antigenic substance, or from ingestion or inhalation of antigen. The former is frequently referred to as *contact dermatitis* and is often an occupational disease resulting from repeated contact with substances such as lacquers, nitrocelluloses, glue, and the like. Ingested antigen not infrequently affects the skin as the shock organ, giving rise to a *non-infectious eczema* in infants and what is often called *neurodermatitis* in the adult.

Urticaria and Angioneurotic Edema. When the skin is the primary shock organ an inflammatory reaction accompanied by some degree of edema results. Urticaria, or hives, is the occurrence of whitish or pink elevations which come and go repeatedly within short periods of time, and is the most common lesion. In angioneurotic edema, or giant urticaria, edema is much more pronounced and the lesions are large pale swellings which cover areas such as the eyelids, lips and genitals. Hives and this edematous kind of lesion are frequently found together and most often result from hypersensitivity to ingested or injected antigen, *i.e.,* foods and drugs.

The foregoing clinical types are characterized by the symptom complex produced, but types of allergy may also be differentiated on the basis of kind and portal of entry of antigen, *viz.:*

Drug Idiosyncrasy. As indicated above, an allergic antigen may be a simple chemical compound, and when this is a drug the condition is a drug allergy or drug idiosyncrasy. The allergic sensitivity is to be differentiated from the sensitivity of a low tolerance for the drug; in the first instance the symptoms are those of the allergic reaction, while in the

[30] Chase: Jour. Exp. Med., 1941, 73:711; Jacobs, Kelley and Sommers: Proc. Soc. Exp. Biol. Med., 1941, 48:639.

second they are produced by the pharmacological action of the drug. The drugs commonly involved are the barbiturate derivatives, salicylic acid compounds, phenolphthalein, opiates, sulfonamides, occasionally the antibiotics, the arsenicals, etc. These are either ingested or injected. The most common reactions are urticaria and dermatitis, and bronchial asthma is less frequent.

Food Allergy. The ingested antigens include food as well as drugs and of the latter strawberries, milk and eggs are the most common offenders. The shock organ in food allergy is usually the skin and the most frequent lesion dermatitis or urticaria, and less often bronchial asthma. In addition, the gastro-intestinal tract is often affected directly with resulting disturbance.

Pollen and Dander Allergies. These antigens are inhaled and the symptoms are almost always those of involvement of the upper respiratory tract, most commonly hay fever and bronchial asthma somewhat less so.

Contact Allergies. This is almost entirely the contact dermatitis noted above and is not only an occupational disease, but not infrequently results from cosmetics, the lacquers such as nail and hair lacquers, and powders containing orris root.

It will be clear from the foregoing that it is difficult if not impossible to generalize to a satisfactory degree the various interrelated forms of allergy, and the source of the difficulty is that they are not fundamentally different. Some workers divide the clinical allergies into two general groups, *atopy* (strange disease) or *atopic allergy* and *non-atopic allergy*. The atopic allergies include the pollen and dander sensitivities resulting in hay fever and asthma, and some of the food and drug allergies. Contact dermatitis and the remainder of the food and drug allergies make up the non-atopic group. The distinction between the two is quantitative rather than qualitative and it is doubtful whether it is of any real validity, but it does have a certain clinical utility. It was first made on the basis of heredity, hereditary predisposition being an important factor in atopy, and it has even been postulated that in atopy initial contact with the antigen is not necessary for the development of the allergic state. On the other hand, in the non-atopic allergies there is almost always a history of contact with the antigen, such as continued exposure to nitrocellulose products in industry, and the hereditary predisposition seems to be of minor importance. There are other correlated characteristics. In atopic allergy the sensitivity is very high, desensitization is difficult and usually only partial at best, skin reactions are marked and specific, and considerable amounts of antibody are demonstrable in the serum. In non-atopic allergy the converse generally holds, *i.e.*, sensitivity is low, desensitization is usually successful, skin reactions are weak and non-specific, and little antibody is demonstrable in the serum. These distinctions are, however, purely relative and of no fundamental significance.

Allergic Antigen and Antibody. As indicated above, the allergic antigens or allergens (sometimes called atopens in the atopic allergies) are frequently non-protein in nature. This is most obvious in the case of the drug idiosyncrasies in which synthetic substances, such as barbiturates, antipyrine and the like, are clearly not contaminated with protein. Similarly, the substances responsible for contact dermatitis such as lacquers are protein-free. The more complex naturally-occurring allergens such as pollens, danders and foods are, of course, not protein-free but the active agent in some pollens is of lower molecular weight, perhaps 5000, than the usual antigenic proteins. There is

no doubt that haptenic substances can produce the allergic reaction in a sen-sitized individual, at least in experimental anaphylactic shock, but the question of sensitization is more difficult. High molecular weight non-proteins can act as complete antigens; for example, sensitization may be induced by pneumo-coccal polysaccharide. It is probable, however, that the relatively simple sub-stances, such as the arsenicals, do not function as sensitizing antigens in them-selves, and it is generally believed that they combine with body protein to give a conjugated complete antigen whose specificity is determined by the haptene, and which functions as the sensitizing antigen. It is not clear, how-ever, whether the shocking antigen is such a conjugated one or whether the haptene alone usually produces shock.

It may be noted here that allergic hypersensitivity to physical stimuli, heat, cold and light, may occur. It is unlikely that such stimuli alter body proteins so that they become isoantigens and such allergies probably do not have an immunological basis.[31]

On intradermal inoculation of soluble antigen, or its application as a patch on the intact skin, the sensitized individual gives a skin reaction characterized by local erythema and the appearance of a wheal. The skin reaction is more readily elicited and more specific in some allergies than in others, and in general is not satisfactory in contact dermatitis and some of the food and drug allergies. This kind of skin test is to be distinguished from tests like the Schick and Dick tests, in which the reaction is produced by diphtheria or scarla-tinal toxin and neutralized by circulating antitoxin, and which have no rela-tion to hypersensitivity.

Antibody is demonstrable in many of the allergies, particularly those grouped under the head of atopy. Its presence may be shown directly in some instances by complement fixation[32] but the usual measure is passive transfer of the sensitivity. If a small amount, 0.1 ml., of serum from a sensitized person is inoculated intradermally into a non-sensitive person and the antigen inoculated intradermally in the same area twenty-four hours later, a positive reaction occurs. The local sensitization may persist for four weeks or more. Such pas-sive transfer cannot be made to guinea pigs, but has been made to rhesus monkeys, and it is probable that a close phylogenetic relationship is essential. This passive transfer was described by Prausnitz and Küstner in 1921 and is known as the *Prausnitz-Küstner reaction*. A reversed passive sensitization, analogous to reversed passive anaphylaxis, may be produced by inoculation of antigen first, and serum twenty-four hours later.[33] In the usual terminology, the antibody which produces passive sensitization is the allergic reagin, but other antibodies which do not sensitize have also been described. The develop-ment of more than one antibody is, of course, to be expected when the antigen contains a mosaic of specificities.

As in the case of anaphylactic shock, the allergic reaction is a consequence of the union of antigen with sessile antibody which results in the liberation of histamine or histamine-like substances. Allergic shock is antagonized by hista-

[31] Bronfenbrenner: Jour. Allergy, 1943, *14:*105.
[32] Hensel and Sheldon: Jour. Lab. Clin. Med., 1941, 26:1586.
[33] Wright and Hopkins: Jour. Path. Bact., 1941, 53:243.

mine antagonists such as epinephrine and ephedrine, and a number of synthetic histamine antagonists such as pyribenzamine (N'-pyridyl-N'-benzyl-N-dimethylethylenediamine), benzhydryl, benadryl, antergan and the like have been of considerable interest in recent years and have some therapeutic promise.

Hypersensitivity in Infection. Bacterial cell substance may act as a sensitizing as well as an immunizing agent, and anaphylaxis may be induced with bacterial protein though usually with much greater difficulty than with highly antigenic proteins such as egg albumin and serum proteins. Sensitization may also occur in experimental and naturally acquired infection, but is highly variable, being especially prone to occur with some bacteria and not with others.

Of the bacterial allergies by far the most thoroughly studied is that developed to the tubercle bacillus. It is demonstrable as a delayed (one to four days) local inflammatory skin reaction, the tuberculin reaction, to preparations of soluble antigen of the tubercle bacillus known as tuberculin. The tuberculin reaction is considered in detail elsewhere (p. 638). This kind of reaction is somewhat different from the immediate wheal type of reaction, with respect to time of development, the nature of the dermal reaction, and in that it is not passively transferable. The generalized response also differs from the immediate shock of anaphylaxis; the inoculation of a tuberculous guinea pig with tuberculin in a dose sufficient to kill does not result in death until after some hours, and on autopsy the site of inoculation is congested, focal glands are swollen and congested, and focal reactions occur about tuberculous lesions which consist of areas of enormous dilatation of the capillaries. This is obviously different from fatal anaphylactic shock in the same animal. The nature of the antigenic stimulus inducing tuberculin-like sensitivity has been shown by Raffel[34] to be a protein combined with a wax fraction of the bacilli, the protein alone giving the usual immune response with the formation of precipitins.

Hypersensitivity is the outstanding immunological response to infection with a number of other microorganisms, and the skin reactions have been of some interest from a diagnostic point of view. In brucellosis, for example, a marked degree of hypersensitivity is developed and a skin reaction, a slightly raised edematous area, appears in about six hours after the intradermal inoculation of preparations of soluble antigen of Brucella. The preparations have been given various names such as abortin (from *Br. abortus*), melitin (from *Br. melitensis*), brucellin and brucellergen. Johnin, a preparation of Johne's bacillus, *Mycobacterium paratuberculosis,* is used in the diagnosis of Johne's disease of cattle. Hypersensitivity occurs with regularity in glanders, and a skin reaction intermediate between the immediate and delayed types follows intradermal inoculation of a preparation of *Malleomyces mallei* designated mallein. Similarly, in chancroid a hypersensitivity develops which is demonstrable as a skin reaction following intradermal inoculation of killed Ducrey's bacillus, and a hypersensitivity occurs in leprosy which results in a positive skin reaction to extracts of leprous tissue termed lepromin. Hypersensitivity to fungi is also not uncommon; the mycids (p. 698) or secondary sterile lesions which occur

[34] Raffel: Jour. Inf. Dis., 1948, 82:267.

in some kind of dermatophytosis are manifestations of hypersensitivity. Infection with *Coccidioides immitis* and *Histoplasma capsulatum* sensitizes and a skin reaction to preparations designated coccidioidin (p. 721) and histoplasmin (p. 724) is demonstrable. Hypersensitivity also occurs in the virus disease, lymphogranuloma venereum (p. 883), and a skin test, the Frei test, with mouse brain antigen or yolk sac culture of the virus, the latter marketed as lygranum, has very considerable diagnostic value.

The Relationship of Hypersensitivity and Immunity.[35] The status of hypersensitivity with respect to effective immunity to infection has been of very considerable interest in relation to tuberculosis. It was early supposed by Koch that the development of tuberculin sensitivity was indicative of immunity and its significance in this respect is indicated by the well known *Koch phenomenon.* If tubercle bacilli are injected subcutaneously into a normal and a sensitized, *i.e.,* infected, guinea pig, the course of the subsequent infection is considerably different. In the normal animal the usual indurated nodule forms, which becomes necrotic to form a persisting necrotic ulcer that persists while the infection spreads via the regional lymphatics, becomes generalized and the animal dies. In the hypersensitive animal, however, an inflammatory reaction occurs but there is no nodule formed; within a day or two the area becomes necrotic and finally sloughs without further spread, and the shallow ulcer heals rapidly. On the other hand, the inoculation of appropriate amounts of tuberculin markedly intensifies the cellular reaction about foci of infection, and the infection tends to spread rapidly. It would seem, therefore, that within limits the hypersensitivity is functional in effective immunity but it may also be of very great disadvantage to the host. The whole question is of particular importance in relation to active immunization against tuberculosis (p. 640).

As indicated earlier, hypersensitivity may take a variety of forms. Perhaps in large part because of clinical differences there has been some tendency to regard the allergies as basically different, not only from anaphylaxis but from one another. It will be clear, however, that there are essentially complete analogies running through all the forms of hypersensitivity and the differences are more apparent than real. They arise from various factors such as the portal of entry of the antigen and its effectiveness in stimulating an immunological response, the shock organ affected, etc. Thus, there is no real difference between the atopic and non-atopic allergies that is not accounted for on the basis of the relative efficacy of the sensitizing antigen—a high degree of sensitivity is associated with ease of demonstration of antibody and interferes seriously with desensitization since it is difficult to give enough antigen to produce an adequate desensitization. Such considerations as these are the basis of the generally accepted belief that hypersensitivity is basically the same regardless of its clinical manifestations.[36]

It is also clear that hypersensitivity is basically an immunological phenomenon, involving the stimulation of antibody formation and the union of antigen and antibody. The paradoxical situation arising from the contrast, say, between the neutralization of toxin by antitoxin, and the acquirement of

[35] Rich: Physiol. Rev., 1941, 21:70.
[36] For instance, see Bronfenbrenner: Trans. Amer. Acad. Ophth. Otolaryng., 1941, Jan.–Feb., 45:30.

toxicity by a bland antigen, is more apparent than real and a consequence of emphasis. Bronfenbrenner[37] has illustrated the relationship by analogy with fire; the warmth, light and other pleasing aspects of a grate fire are also present when the house burns down, both involve precisely the same mechanism, yet the emphasis on the consequences of the combustion is entirely different in the two cases.

[37] Bronfenbrenner: Amer. Rev. Tuberc., 1937, 36:293.

THE STAPHYLOCOCCI

The bacteria most commonly found in boils, abscesses, carbuncles and similar suppurative processes in man belong to the group of staphylococci. The presence of staphylococci in pus was first shown by Pasteur[1] and later by Ogston.[2] Micrococci were obtained in pure culture by Becker[3] in 1883, but their causal relation to the suppuration of wounds and to osteomyelitis was first brought out by the work of Rosenbach[4] in 1884.

Morphology and Staining. The spherical cells are generally aggregated in loose, irregular masses which have been likened to clusters of grapes, and have given the generic name to this organism. In a preparation made directly from pus or from a pure culture, not only irregular clusters of cells can be observed but also tetrads, pairs and short chains of cells. It is, then, often difficult to determine from stained preparations whether only true staphylococci are present or whether there is an admixture of streptococci or other forms. The irregular clusters are most consistently observed in preparations from cultures on agar media, preparations from broth cultures showing, as a rule, greater dispersion of the cells.

The staphylococci do not form spores, and motile varieties are very rarely observed. The ordinary aniline dyes stain the cells readily, and the great majority of staphylococci are gram-positive. The dimensions of the individual cocci vary within narrow limits, the diameter of the cells ranging between 0.7 and 0.9 μ.

The growth on agar media is abundant, opaque and glistening, and the individual colonies are circular with entire edges. Pigment may be formed, a golden yellow in the case of *Staphylococcus aureus* and lemon yellow in the case of *Staphylococcus citreus,* but it is absent and the growth appears white in the case of *Staphylococcus albus* and other less common species.

Physiology. The optimum temperature is 28° to 37° C., the latter in the case of those species found in association with man and lower animals; but growth can also take place at temperatures as high as 42° C. and as low as 8° to 9° C. The best known species, sometimes designated the aerobic staphylococci, grow best in the presence of oxygen and to some extent under anaerobic conditions; certain other species are, however, strict anaerobes. The staphylococci in general are not fastidious in their nutritive requirements and grow readily upon the ordinary nutrient media. Investigation of *Staphylococcus*

[1] Pasteur: Bull. de l'Acad. de Med., 1880, 9:447.
[2] Ogston: Brit. Med. Jour., 1881, 1:369.
[3] Becker: Deut. med. Wchnschr., 1883, 9:665.
[4] Rosenbach: *Mikroorganismen bei d. Wundinfektionskrankheiten.* Wiesbaden. 1884.

aureus has shown that, although frequently requiring amino acids at first, this species may in time be grown with an ammonium salt as a sole source of nitrogen.[5] Both nicotinic acid and thiamine (aneurin, or vitamin B_1), together with an organic source of sulfur are, however, required,[6] and uracil[7] must be supplied for anaerobic growth. Growth in such synthetic solutions is greatly improved by the addition of biotin (bios II_B, vitamin H), and this substance may also be required by this microorganism.[8]

In the fermentation of glucose, lactic acid is the predominating end product (77 to 91 per cent), together with small quantities of ethyl alcohol and carbon dioxide.[9] The golden yellow pigment which distinguishes the *aureus* variety from other staphylococci is formed most abundantly upon carbohydrate media
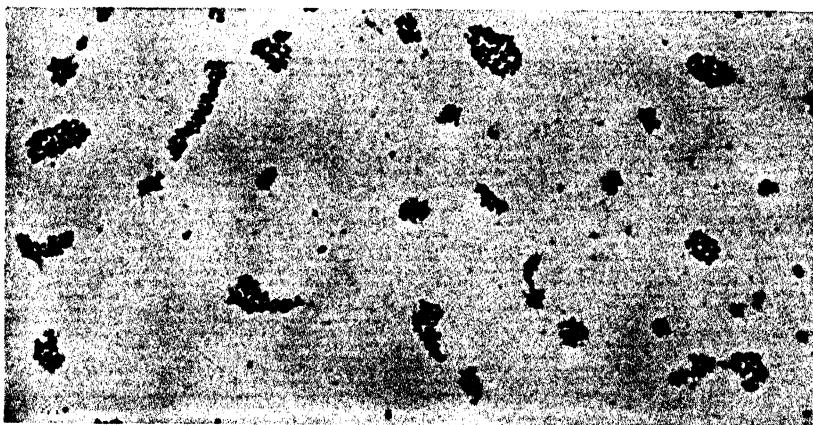
Fig. 42. *Staphylococcus aureus* from pure culture. Note the characteristic clusters of the cocci. Fuchsin; $\times$ 1050.

or blood serum in the presence of free oxygen. The pigments produced by the chromogenic staphylococci are probably lipochromes. A specific gelatin-liquefying enzyme or gelatinase is formed in the majority of gelatin and broth cultures and has been separated from the cultures by filtration. Other enzymes, such as rennin and maltase, are produced under suitable conditions. Dextrose, lactose, sucrose, maltose, glycerol and mannitol are usually fermented with acid but no gas, while raffinose, salicin and inulin are not attacked nor is starch hydrolyzed. Indol is not produced and nitrate reduction is variable. Milk inoculated with staphylococci is usually coagulated by the acid resulting from fermentation, but the precipitated casein remains, as a rule, undissolved.

The thermal death point is not constant, different strains appearing to vary greatly in their resistance, some succumbing only after thirty minutes' exposure to a temperature of 80° C., while others are destroyed at a much lower

[5] Gladstone: Brit. Jour. Exp. Path., 1937, *18*:322.
[6] Knight: Biochem. Jour., 1937, *31*:966.
[7] Richardson: Biochem. Jour., 1936, 30:2184.
[8] Kögl and van Wagtendonk: Rec. trav. chim., 1938, 57:747; Porter and Pelczar: Science, 1940, *91*:576.
[9] Friedemann: Jour. Biol. Chem., 1939, *130*:61.

temperature. Considerable resistance is displayed toward drying, experiments showing a retention of vitality for many days and even months in cultures dried upon silk threads and desiccated over calcium chloride. Toward the chemical substances ordinarily used as disinfectants the staphylococci also exhibit more than the average resistance. They are, in general, among the hardiest of the non-spore-forming bacteria.

Toxins.[10] A variety of toxic substances are produced by staphylococci, including hemolysins, leucocidin, coagulase, fibrinolysin, spreading factor (Duran-Reynals factor), skin-necrotizing substance, a lethal factor and enterotoxin.

Hemolysins. The pyogenic staphylococci are almost invariably β-hemolytic on blood plates and produce filterable hemolysins in broth culture, while the saprophytic forms are less frequently hemolytic. The filterable hemolysins

Fig. 43. Colonies of *Staphylococcus aureus* on nutrient agar. Twenty-four-hour culture; × 3.

(staphylolysins) are of two types, one lysing red cells upon incubation (α-lysin) and the other (β-lysin) only after holding in the icebox following preliminary incubation—the so-called "hot-cold" lysis (p. 207).

Other Toxins. The ability of many pyogenic staphylococci to coagulate citrated plasma, lyse fibrin clots, kill leucocytes and increase the permeability of the skin may be shown by appropriate techniques. Sterile filtrates from broth cultures produce necrosis upon intradermal injection—the so-called skin-necrotizing factor—and almost immediate death when injected intravenously into rabbits. Whether these and other effects are due to the activity of separate substances or are various manifestations of the activity of a single substance is uncertain. It is definitely established that more than one hemolysin is produced and that the enterotoxic substance, effective *per os* and of considerable food-poisoning significance (p. 272), is not identical with the other activities. More than one toxic substance is produced, then, but probably some of these produce more than one effect. In general, toxin production is a property of the pathogenic staphylococci, usually of the *aureus* variety.

[10] *Cf.* Blair: Bact. Rev., 1937, 3:97.

Variation. Like other bacteria, the staphylococci dissociate, and both rough and G colonial types have been described.[11] These have not been thoroughly studied, however, and are not well known.

Classification. The genus *Staphylococcus* was formerly one of four which made up the family *Micrococcaceae* but the generic name has been dropped by Bergey (1948) in favor of *Micrococcus*. The staphylococci are roughly divisible into two types, the one designated as aerobic, though the bacteria are in fact facultative anaerobes, and the other which is strictly anaerobic. The common species comprising the former group, which is by far the better known, are the pigmented forms *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*), which is golden yellow and generally pathogenic, and *Staphylococcus citreus* (*Micrococcus citreus*), which is lemon yellow and a saprophyte, together with the non-pigmented *Staphylococcus albus* (*Micrococcus pyogenes* var. *albus*), and *Staphylococcus epidermis* (*Micrococcus epidermis*). The non-pigmented forms are saprophytic or only feebly pathogenic and are commonly not distinguished but lumped under the name of *albus*. Still other species form pink to reddish brown pigments. There are several species of strict anaerobes, *Staphylococcus aerogenes*, *Staphylococcus asaccharolyticus*, *Staphylococcus anaerobius*, *Staphylococcus niger* and *Staphylococcus grigoroffi*. These anaerobic species are inhabitants of the body cavities, and the first is occasionally the cause of puerperal fever.

Physiological Differentiation. The validity of these species is, however, open to serious question. The differentiation of the better known aerobic species on the basis of pigment production is of doubtful value, for this characteristic is variable. Recently isolated pyogenic cocci forming a rich golden pigment lose this property on continued cultivation on artificial media and become identical with the *albus* variety. No biochemical tests serve to sharply differentiate these bacteria from one another. The strains that produce white colonies are, as a rule, less active in gelatin liquefaction and fermentative power, and hence have been sometimes regarded as weakened relatives of the biochemically more vigorous golden pigmented types. The *albus* strains are also as a rule only feebly pathogenic. Dextrose, maltose and glycerol are fermented by nearly all *aureus* strains and by a consistently lower proportion of *albus* strains, lactose and mannitol by about four-fifths of the *aureus* and by about one-third (mannitol) to two-thirds (lactose) of the *albus* strains.[12] In general, the types most commonly isolated from air, dust and other sources outside of the human body are white staphylococci, while those found associated with pathologic conditions are *aureus* strains. The general opinion is that the staphylococci form a closely graded series from the pigmented, hemolytic, gelatin-liquefying, pathogenic, actively fermenting strains to those that are unpigmented, feebly pathogenic and less actively hemolytic, liquefying and fermenting.

Immunological Differentiation. Differentiation of the staphylococci on the basis of agglutination reactions has as yet been unsatisfactory. It has been shown,[13] however, that two immunological types may be differentiated by pre-

[11] Hoffstadt and Youmans: Jour. Inf. Dis., 1932, *51*:216; Bigger, Boland and O'Meara: Jour. Path. Bact., 1927, *30*:261; Swingle: Jour. Bact., 1935, *29*:467.
[12] Dudgeon and Simpson: Jour. Hyg., 1927, *27*:160.
[13] *Cf.* Julianelle and Wieghard: Jour. Exp. Med., 1935, *62*:11, 23, 31.

cipitation tests with specific carbohydrates extracted from the microorganisms. One of these types, designated type A, is composed for the most part of strains isolated from pathogenic sources, while the other, type B, comprises strains of non-pathogenic origin. The majority of type A strains ferment mannitol, while those of type B do not.

The Coagulase Test. The ability of staphylococcus strains to coagulate plasma appears to be associated with pathogenicity in that the majority of strains isolated from pathologic processes are coagulase-positive while the saprophytic strains are usually coagulase-negative. It has been suggested that the staphylococci be divided into pathogenic and non-pathogenic types on this basis, and in recent years the coagulase test has been generally used.[14] It has been found also that coagulase-positive strains clump rapidly when mixed with fresh human plasma; the CO_2-soluble fraction of fibrinogen seems to be the most important part of the plasma in effecting immediate clumping.[15] The slide test[16] for clumping, carried out by mixing fresh, undiluted human plasma with heavy bacterial suspension, is a simple and rapid means of differentiation.

Pathogenicity for Man.[10] Experimental evidence and that of comparative pathology show that man is more susceptible than the ordinary laboratory animals to staphylococcus infection. Garré[17] inoculated himself by rubbing a pure culture upon the uninjured skin of the forearm, with the result that a series of carbuncles was produced, seventeen scars remaining to testify to the success of the experiment. The penetration of the cocci into the deeper layers of the intact skin, probably through the sweat ducts or at the base of the hair follicles, is a fact of considerable significance. The positive occurrence of such penetration seems well established, and the negative observations of some authors may well be referred to differences in the virulence of the strains employed or to other experimental discrepancies.

The demonstration that staphylococci have power under certain circumstances to penetrate the skin, taken together with their practically constant presence upon the skin itself, serves to explain the multiplicity of human affections with which these microorganisms are found associated. A momentary weakness on the part of the tissues in almost any locality may lead to a rapid local invasion, followed by the production of a simple boil or by a more or less extensive carbuncular condition. Septicemia and pyemia sometimes result through the introduction of staphylococci into the lymphatics or the blood stream from a local abscess. The initial lesion may be trivial in character. In the series of 122 cases studied by Skinner and Keefer[18] the case fatality rate was 82 per cent. Rapidly fatal bacteremia may occur without metastatic abscesses or metastatic abscesses may be produced, or the bacteremia may clear but leave focal abscesses and the infection still be fatal.

Staphylococci are not only found frequently in all parts of the body in secondary and mixed infections, but they are also primarily responsible for a variety of specific pathologic conditions and for injury to particular organs.

---

[14] For details see Fisk: Brit. Jour. Exp. Path., 1940, 21:311; Gillespie: Med. Res. Council (Great Britain) Monthly Bull., Emergency Pub. Health Lab. Service, 1943, 2:19.

[15] Berger: Jour. Path. Bact., 1943, 55:435.

[16] *Cf.* Cadness-Graves, Williams, Harper and Miles: Lancet, 1943, i:736.

[17] Garré: Fortschr. d. Med., 1885, 3:165.

[18] Skinner and Keefer: Arch. Int. Med., 1941, 68:851.

Many lesions and diseases of the skin have been attributed to staphylococci. In the case of some of these it has been claimed that special varieties or races are concerned, but the characters said to distinguish these from the ordinary *Staphylococcus aureus* or *albus* are not, as a rule, of differential value.

A considerable majority of all attacks of acute osteomyelitis and periostitis are due to staphylococci, which appear to have a special predilection for the tissues of the osseous system.

Suppurative inflammation, in whatever part of the body it may occur, is usually associated with the presence of staphylococci either in pure or mixed cultures. Sometimes when found in a mixed infection they are doubtless the original exciting cause; in other cases they may have arrived at the seat of trouble only after a primary invasion by some other bacterium. In a given instance it may be impossible to determine the precise sequence of events.

Staphylococcus infection of the lung sometimes occurs, and the resulting bronchopneumonia is often fatal. Out of about 800 patients with pneumonia treated at the Hospital of the Rockefeller Institute in New York City from 1913 to 1918, 13 were infected with staphylococci, and 10 of the 13 died. Under certain conditions, as during the 1918 influenza epidemic at Camp Jackson, *Staphylococcus aureus* may play an important part in the pneumonia complicating primary infections. Chickering and Park[19] found that in 49 per cent of 312 postmortem lung cultures this microorganism was present either alone (92 cases) or in association with other bacteria. Similarly, Gaspar[20] found that of 144 fatal cases of pneumonia cultured at autopsy, 38 were caused by staphylococci. In this series about two-thirds of the staphylococcus pneumonias occurred in the first decade of life. The presence of staphylococcus in the lungs is usually interpreted as a secondary invasion in the train of some primary exciting agent.

Staphylococcus food poisoning has been discussed in a previous chapter (Chap. 11).

Bacteriological Diagnosis. The isolation of staphylococci from pathologic material or from foods suspected in outbreaks of food poisoning is a simple matter. Blood agar is the medium of choice since the pathogenic strains are usually hemolytic *Staphylococcus aureus,* and the hemolytic character is apparent on this medium. Examination of gram-stained smears from golden-yellow hemolytic colonies will show the characteristic morphology. Colonies may be picked for the coagulase test (see above) but fermentations are not significant and immunological typing is desirable only in special studies. The ability to form enterotoxin in the case of food poisoning strains may be demonstrated by feeding sterile filtrate of subcultures incubated in 25 per cent CO_2 to human volunteers in amounts of 2 to 5 ml., or to rhesus monkeys by stomach tube in 25 to 50 ml. amounts. The kitten test for enterotoxin is not reliable.

Pathogenicity for Lower Animals. The rabbit has proved one of the more favorable animals for experimentation, intravenous injection of broth cultures being the most successful mode of infection. A moderately virulent strain kills an average-sized rabbit in four to eight days after injection of 0.1 ml.

[19] Chickering and Park: Jour. Amer. Med. Assn., 1919, 72:617.
[20] Gaspar: New York State Jour. Med., 1941, 41:834.

of a twenty-four-hour broth culture. On autopsy, minute abscesses are found in various internal organs, most commonly in the kidney (particularly in the cortex) and in the walls of the heart. Under ordinary conditions of experiment with healthy adult rabbits the bone marrow and periosteum are rarely seriously affected. In young animals, however, several workers have claimed to have evoked typical osteomyelitis by intravenous injection of staphylococcus cultures. It is perhaps questionable whether in these cases the processes in the affected tissues are strictly comparable with natural osteomyelitis in man. The injection of cultures into a rabbit suffering from a fractured bone or an injured periosteum produces a more characteristic chain of events, and one that closely resembles the course of human osteomyelitis. Rabbits are relatively insusceptible to intraperitoneal inoculation with staphylococci. Artificial inoculation

Fig. 44. *Micrococcus tetragenus;* smear from pure culture stained with fuchsin. Note the relatively large size of the cells and the typical tetrad arrangement with the irregular clumps tending to be made up of tetrads. × 1800.

of the eye, on the other hand, succeeds readily, although natural eye infection is never observed. Feeding experiments with staphylococci do not produce infection. White mice are sometimes used for inoculation experiments, but are less uniformly responsive than rabbits; guinea pigs are relatively resistant, rats and pigeons highly so.

Cases of spontaneous staphylococcus infection among domestic animals, while not so common as in man, are not unknown. In horses and cattle *Staphylococcus aureus* has been found associated with pathologic processes and conditions similar to those that it produces in man. Staphylococcus mastitis in cattle is not uncommon.[21] Some observers believe that they have discovered special species of staphylococci in certain animal affections, such as "*Staphylococcus bovis*" (in cattle) and "*Staphylococcus hemorrhagicus*" (in sheep), but such species are of doubtful validity. Typical strains of *Staphylococcus aureus* and *albus* have been isolated from spontaneous abscesses in birds.

[21] *Cf.* Minett: Jour. Comp. Path. Therap., 1937, 50:101; Plastridge, Anderson, Williams and Weirether: Storrs Agr. Exp. Station Bull. No. 231, 1939.

Immunity. An active immunity may be developed against staphylococcal infection by immunization with vaccines. In the rabbit the use of killed suspensions followed by living attenuated bacteria occasionally results in infection, and the inoculation of living microorganisms is somewhat dangerous. Immunization of human beings is, therefore, confined to the use of killed suspensions. The immunity so induced is in part antibacterial and in part antitoxic if the toxic products of the microorganism are included in the vaccine. The antibacterial immunity appears to be essentially a stimulation of the phagocytic mechanisms; the rate of phagocytosis is remarkably increased, and the activity of the phagocytic cells is of prime importance in combating infection with these bacteria. According to Valentine and Butler[22] antileucocidin is of considerable importance in the immunity. Antibacterial sera will protect untreated animals against infection, and the acquired immunity is associated with an increase in the amount of opsonin.

Fig. 45. Colonies of *Micrococcus tetragenus* on blood agar. Twenty-four-hour culture. × 3.

The therapeutic use of vaccines in the treatment of boils and carbuncles and chronic furunculosis in man has met with some success. There is good evidence that the autogenous staphylococcus vaccine (the strain cultivated from the patient) is more efficacious than the ordinary stock vaccine.

Considerable interest has attached to the antitoxic aspect of antistaphylococcus immunity. Toxoid, prepared by treating staphylococcus culture filtrates with formalin, has been used in producing an active antitoxic immunity, and a number of attempts have been made to demonstrate the efficacy of antitoxic sera. The therapeutic use of toxoid has given encouraging results in some instances, and it is of interest that the antileucocidin appears to be of particular significance.[23] The value of antitoxic sera is uncertain. Their use, either through parenteral inoculation or local application, has not led to unequivocal results though in a number of instances very favorable results have been re-

[22] Valentine and Butler: Lancet, 1939, *ii*:973.
[23] *Cf.* Flaum: Acta. Path. Microbiol. Scand., Suppl. 35, 1938.

ported.[24] The antitoxin, it may be noted, is standardized in terms of anti-hemolysin, one unit being that amount which will neutralize 200 minimum hemolytic doses of toxin; in consequence the toxin is sometimes differentiated into α-toxin and β-toxin, referring to the α- and β-lysins.

OTHER MICROCOCCI

A variety of micrococci, both pigmented and otherwise, have been described, most of which are saprophytic forms found in water and elsewhere in nature. A well-known representative of this group is *Sarcina lutea,* a coccal form producing a bright yellow pigment which derives its generic name from a ten-tendency to form cubical packets of eight cells.

Micrococcus tetragenus (*Gaffkya tetragena*) is a parasitic coccus frequently found on the mucous membranes of the upper respiratory tract. It was discovered by Gaffky[25] in the pulmonary cavities in phthisis, and has been found in pure culture in abscesses in animals and man, and often occurs in the healthy mouth. Morphologically *Micrococcus tetragenus* is distinguished by its occurrence in tetrads or groups consisting of four small oval cocci. It is gram-positive. In cultures the sheet-like arrangement is not always seen, but in the animal organism the flat tablets occur uniformly, and a rather heavy capsule surrounds the tetrad. On agar a confluent rough, elevated white growth is produced. On potato a thick, white, slimy growth occurs. Gelatin is not liquefied; milk is coagulated. Growth is slow and occurs at 20° and at 37° C., though better at the higher temperature.

White mice inoculated with *Micrococcus tetragenus* succumb to a rapidly progressing septicemia. Guinea pigs and rabbits usually show only a local affection. House mice and rats are relatively resistant. Fornaca[26] has reported a case of septicemia in man in which *Micrococcus tetragenus* was present in pure culture in the blood. It is not uncommonly found in suppurations of the mouth and neck. It is also found in the empyema following pneumonia and in the pus of war wounds.

This microorganism is probably of low-grade virulence, and unable, as a rule, to invade the human tissues except when the resistance is lowered by some depressing influence, especially of the kind caused by the invasion of some other bacterium.

[24] *Cf.* Kleiger and Blair: Arch. Surg., 1943, 46:548.
[25] Gaffky: Mitt. a. d. k. Gesund., Berlin, 1881, 1:1.
[26] Fornaca: Rif. Med., 1903, 19:309.

THE STREPTOCOCCI

The streptococci make up a relatively large group of pyogenic coccus forms characterized by an arrangement of the cells in chains. They are responsible for a variety of diseases of man, certain diseases of lower animals, and some are saprophytes found in milk and milk products. They were early observed in the pus formed in suppurative inflammatory conditions, and their frequent presence and pathologic significance were first emphasized by Ogston, by Fehleisen and by Rosenbach in the early 1880's. It is now well known that, in addition to the more virulent pathogenic forms, relatively harmless parasitic streptococci are more or less constantly present in the human throat and in
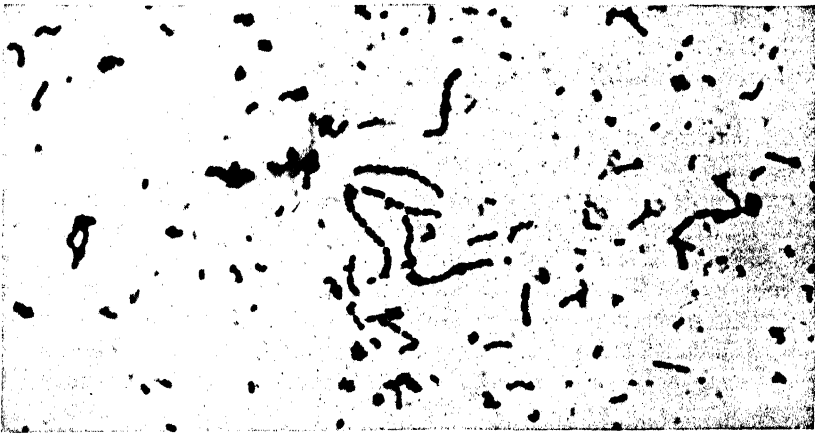
Fig. 46. *Streptococcus pyogenes.* Recently isolated scarlet fever strain. Note the tendency to diplococcus arrangement in the chains. Fuchsin; × 1050.

the intestinal tract which assume a pathogenic role only under circumstances in which normal resistance is markedly reduced, and which may be regarded, for all practical purposes, as a portion of the normal flora of the human body.

Morphology and Staining. Like staphylococci, individual streptococci are spherical and 0.8 to 1.0 μ in diameter. Some variation in size results from the character of the culture medium, and the individual cells are frequently appreciably smaller when the cultures are grown under anaerobic conditions. Smaller varieties, 0.4 to 0.8 μ in diameter, whose size is apparently a constant character, have been described. The typical streptococcus divides in only one plane, and the tendency of the cells to remain united results in the development of the characteristic chains that give these organisms their generic name.

355

This tendency is apparently more pronounced between daughter cells following the first cell division, and the chains frequently have the appearance of chains of diplococci, with pairs closer to one another than to adjacent pairs; this is apparent in Fig. 46. The firmness of the attachment is to some degree a strain characteristic and some strains appear as relatively long chains while others show little more than two pairs of diplococci. Earlier workers attached some importance to the length of chains formed and differentiated a supposedly more virulent *Streptococcus longus* and a less virulent *Streptococcus brevis*. Other morphological and biochemical characteristics were thought to be associated. It has long been clear, however, that this distinction has little meaning in spite of the fact that freshly isolated streptococci from pathologic processes usually form chains of more than eight cells while those normally present in the mouth and throat usually develop only short chains. For instance, longer chains are generally formed during growth in liquid media; *Streptococcus lactis,* a common inhabitant of milk, forms very long chains; and short-chain streptococci are not infrequently isolated from pathologic conditions. The formation of chains of cells is in no sense absolute, however, and on miscroscopic examination of a smear of typical streptococci, single cells, pairs of cells, and occasional aggregates resembling staphylococci are found.

Streptococci are not motile under ordinary conditions of observation, but motile forms are described with some frequency, and the precise status of their motility is not clear. The majority of strains are encapsulated, and in some the capsular material consists of hyaluronic acid, the substrate of hyaluronidase or invasin (p. 212). So far as is known, these bacteria do not form spores, and the formation of pigment is relatively rare.

They stain readily with the usual bacterial stains. Almost all of the strains isolated from pathologic processes in man are gram-positive, but gram-negative streptococci are found with some frequency, more commonly in suppurative conditions in lower animals than in man.

Streptococcus colonies on agar media are usually quite small, translucent, convex, entire, and slightly granular, but colonial differences among variants are well known (*vide infra*). The growth on agar plates may be confluent if too heavily inoculated, but these bacteria have a tendency to remain in discrete colonies.

Physiology. As a group, the streptococci grow over a relatively wide temperature range, 10° to 42° C. Those of the pyogenic group, which is made up of human and animal parasites, have an optimum at 37° C. and are relatively restricted in range, those of the lactic group grow over 10° to 37° C., and the viridans group grows from 37° to 42° C. and includes one thermophilic species which grows at 50° C. The majority of streptococci are facultative anaerobes, but there are a few obligate anaerobic varieties.

The streptococci are among the more fastidious of bacteria with respect to nutritive requirements. They will usually not grow on meat extract media, and growth is ordinarily poor even on infusion media but may be somewhat improved by the inclusion of phosphate buffer (M/30) and a small amount, perhaps 0.1 per cent, of glucose. For the most part, however, infusion media enriched by the addition of 10 per cent defibrinated blood, ascitic fluid and similar substances are used and a medium such as blood agar is completely

satisfactory for routine culture of the pathogenic forms. Many strains are hemolytic on blood agar, some showing the clear zones of β hemolysis (see Fig. 47), and others a zone of greenish discoloration or a hemolysis indistinguishable from that produced by the pneumococcus (Fig. 52). Growth also occurs in milk which is curdled by some species due to the fermentation of lactose. Streptococcus cultures may be preserved in serum broth or agar or infusion media gelatin stabs in the refrigerator.

The relatively complex nutritive requirements of these bacteria have been defined to a considerable degree. Most strains require glutamine, riboflavine, pantothenic acid, pyridoxine, nicotinic acid and biotin, together with thirteen or fourteen amino acids. According to Wilson[1] the streptococci of group A also require nucleic acid derivatives. Some strains have been cultivated in chemically defined media[2] and Bernheimer and Pappenheimer[3] have developed a hydrolyzed casein medium supplemented with amino acids, salts, glucose and vitamins of the B group which supports good growth of hemolytic streptococci.

A wide variety of sugars is fermented and a number of polysaccharides are hydrolyzed. The chief fermentation product of glucose is lactic acid, and small amounts of formic and acetic acids and ethyl alcohol are formed.[4] The hydrolysis of sodium hippurate and polymers such as inulin, starch and dextrin has some differential significance, together with the fermentation of lactose, sorbitol, glycerol, mannitol, maltose, sucrose and raffinose. Some strains liberate relatively large amounts of ammonia from peptone and this characteristic also has some differential value. With rare exceptions, inulin is not fermented nor are the streptococci dissolved in ox bile or a 10 per cent solution of bile salt; these characteristics have considerable practical importance in that they serve to differentiate the a hemolytic or green streptococci from the pneumococci.

The Formation of Toxic Substances. The streptococci form no endotoxin, *i.e.*, the cell substance is only mildly toxic on parenteral inoculation, and no true exotoxin unless the scarlatinal toxin be included in the exotoxins. A number of toxic substances of agressin-like character which contribute to the invasive qualities of the pathogenic streptococci are found in culture fluid. These include hemolysins, leucocidin, fibrinolysin, hyaluronidase and the erythrogenic or scarlatinal toxin, and there is, in addition, a substance lethal for mice whose relation to the other toxic substances is not clear.

Two kinds of filterable hemolysin produced by streptococci were described by Todd.[5] They differ in that one, called *streptolysin S*, is sensitive to treatment with heat or acid, and the other which is designated *streptolysin O* is inactivated by oxygen, *i.e.*, is inactive in the oxidized state, and the activity may be re-

---

[1] Wilson: Proc. Soc. Exp. Biol. Med., 1945, 58:249.

[2] Hutchings and Wooley: Science, 1939, 90:41; McIlwain *et al.*: Biochem. Jour., 1939, 33:223; Hutner: Jour. Bact., 1938, 35:429; King, Gary and Farrell: Jour. Bact., 1938, 36:837; Subbarow and Rane: Jour. Amer. Chem. Soc., 1939, 61:1616; Wooley: Jour. Exp. Med., 1941, 73:487.

[3] Bernheimer and Pappenheimer: Jour. Bact., 1942, 43:481, 495.

[4] Friedmann: Jour. Bact., 1938, 35:527; *ibid.*, Jour. Biol. Chem., 1939, 130:757; Smith and Sherman: Jour. Bact., 1942, 43:725.

[5] Todd: Jour. Path. Bact., 1938, 47:423; Herbert and Todd: Brit. Jour. Exp. Path., 1944, 25:242.

stored by treatment with mild reducing agents such as sulfite. Both hemolysins are extremely labile at 37° C. and disappear rapidly after the first few hours of incubation. There is some evidence that the leucocidin of streptococci is identical with streptolysin O. The latter has been studied in some detail by Bernheimer,[6] who has found that it has a cardiotoxic action on the isolated frog heart, and appears to be associated in some manner with the mouse lethal factor. The fibrinolysin (p. 211), acting as an activator of an inactive serum protease, is possibly associated with the invasive character of the pathogenic streptococci in facilitating the spread of infection through the barrier of fibrin clots.

The relation of hyaluronidase (p. 212) to the invasive properties of the streptococci is less clear than once thought. In general, as with many other pathogenic bacteria, the presence of capsules is associated with virulence and among the streptococci the capsular substance is frequently hyaluronic acid. The addition of hyaluronidase to suspensions of encapsulated streptococci thus denudes them of capsules and they are more readily phagocytosed and less virulent.[7] This factor, then, assumes an anomalous position with regard to its contribution to the invasive properties of streptococci.

The erythrogenic toxin is a substance which gives rise to a marked local erythema upon intradermal inoculation in man, and in larger amounts produces a generalized erythematous rash. A skin reaction may be produced in the rabbit but in general laboratory animals are highly or completely resistant to it. The lethal dose for the rabbit is very large, 5 to 10 ml. of unconcentrated filtrate. This toxin is responsible for the rash of scarlet fever and is known as scarlatinal toxin, or Dick toxin after its discoverers. It differs from the classic exotoxins in that it is relatively heat-resistant and some toxicity still remains after boiling for thirty minutes (see also p. 375). It is antigenic and stimulates the production of specific antitoxin but not to the high titers readily obtained for diphtheria and tetanus antitoxin.

The formation of the foregoing substances by the streptococci is a characteristic of the group rather than of all strains of pathogenic streptococci. Thus all group A streptococci do not produce erythrogenic toxin, and those that do not are incapable of causing scarlet fever. In general they are associated with virulence in that avirulent varieties are frequently non-hemolytic, non-fibrinolytic and the like while those found in pathologic conditions and showing high virulence under experimental conditions are hemolytic, fibrinolytic, etc.

Another factor associated with the ability of streptococci to produce disease is the development of a hypersensitivity to the cell substance of these bacteria during and following infection. Subsequent infections, then, result in allergic phenomena which may be of very considerable importance in the disease produced. Thus, it seems probable that hypersensitivity plays a part in rheumatoid disease and arthritis (*vide infra*) of streptococcal etiology.

Variation. Alterations in the morphology of individual streptococci are frequently observed in old cultures, with cells swollen to several times normal size. These and other changes in aging cultures have been interpreted by some workers as indicative of a complex life cycle, but it is more likely that

[6] Bernheimer and Cantoni: Jour. Exp. Med., 1945, *81*:295, 307; *ibid.*, 1947, 86:193.
[7] See Kass and Seastone: Jour. Exp. Med., 1944, 79:319.

such abberant morphology is that of involution forms, *i.e.*, is degenerative in nature.

Dissociative changes in colonial morphology are well known and have been described by Todd[8] and by Dawson, Hobby and Olmstead.[9] It was shown by the former that a form designated as matt is the virulent form and distinct from the usual smooth and rough colony types. The latter workers described a mucoid colony type which was still different. There are, then, four recognized colonial types: smooth, rough, mucoid and matt. The conversion from mucoid or matt to rough or smooth corresponds to the usual S→R dissociative change. The type-specific M protein is present in all but the rough form. Other immunological variation, not correlated with colonial form, may occur also, for there is some evidence that the agglutinative types are sometimes unstable, and that on occasion the group-specific polysaccharide may be lost.

Variation in hemolysis is commonly reported in which β hemolytic strains give rise to non-hemolytic or α hemolytic variants. The alteration in hemolysis is to some degree an environmental effect in that anhemolytic variants may be hemolytic under anaerobic conditions, suggesting an inactivation of oxygen-labile hemolysin rather than failure to produce it; similarly α hemolytic variants may be made β hemolytic by including catalase in the medium or omitting reducing sugar since the latter appears to inhibit hemolysin production by some strains of streptococci. Streptolysin O is generally regarded as responsible for blood plate hemolysis, but anhemolytic variants of β hemolytic strains have been observed which continue to form streptolysin O in liquid culture.

Streptococci, like other bacteria, may become resistant to the action of chemotherapeutic drugs in the laboratory. Drug-fast strains may also be found in naturally occurring infections but whether these arise by a process of selection or by *in vivo* adaptation in the presence of drugs during therapy is not clear (p. 178). Whatever the mechanism, the widespread, and often indiscriminate, use of such drugs through the general availability of sulfathiazole tablets, penicillin tablets and the like, has been regarded by many as undesirable because of this possible consequence. The development of just such a situation occurred during World War II through the use of sulfadiazine as a prophylactic for the control of streptococcus infection in naval training camps.[10] The sequence of events was as follows: Since preliminary studies indicated that streptococcal infection among trainees could be controlled by prophylactic sulfadiazine, all personnel at one training center were placed on such a prophylactic regime on March 1, 1944. Following its institution streptococcal infection increased but this was associated with a country-wide increase and tests made at the time indicated that no sulfa-fast streptococci were present at the center. At that time *Str. pyogenes* Type 19 was responsible for about 28 per cent of the infections. The high incidence of infection continued and by May Type 19 was responsible for 95 per cent of cases of streptococcal disease. At that time it was found that a high percentage of strains of Types 19 and 17 isolated were sulfa-fast, and Type 3 had also become drug-fast. These drug-fast strains were

[8] Todd: Brit. Jour. Exp. Path., 1928, 9:91; Jour. Exp. Med., 1932, 55:267.
[9] Dawson, Hobby and Olmstead: Jour. Inf. Dis., 1938, 62:138; also Morton and Sommer: Jour. Bact., 1944, 47:123.
[10] Jour. Amer. Med. Assn., 1945, 129:921; Damrosch: *ibid.*, 1946, 130:124.

highly communicable and formed scarlatinal toxin. The drug-fast Type 19 appeared at other training centers coincident with the transfer of personnel. By July prophylactic sulfadiazine had been discontinued in all but two of five primary training centers where it was retained for half the personnel for experimental purposes. Subsequent analysis showed that these two centers had the highest admission rates for patients with throat cultures positive for hemolytic streptococci, and the incidence of scarlet fever was much higher in the treated groups. Under the circumstances prophylactic sulfadiazine apparently increased the incidence of streptococcal infection, and it was suggested that the drug-fast strains were actually stimulated by small amounts of the drug or that the drug inhibited selectively normal flora of the throat and nose which ordinarily had some antibiotic effect on streptococci. Not only did the drug-fast strains spread through personnel from one locality to another,
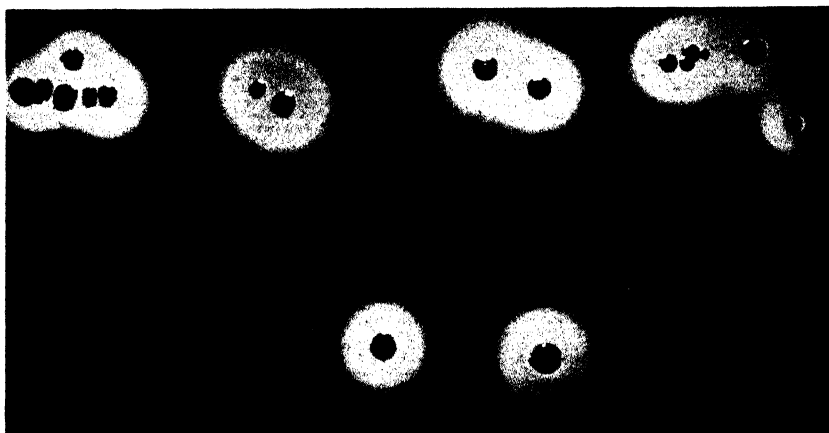
Fig. 47. *Streptococcus pyogenes.* Pure culture on blood agar showing β hemolysis. $\times$ 5.

but drug-fast Type 19 *Str. pyogenes* appeared in the civilian population coincident with the return of large numbers of armed service personnel to civilian life.[11] It will be clear that the development of resistance to chemotherapeutic drugs can be a matter of very considerable practical importance, especially with virulent pathogenic bacteria as ubiquitous as the streptococci.

Classification.[12] The differentiation and identification of the streptococci is a matter of very considerable practical importance because of their etiological relation to a number of widespread diseases of man and domestic animals, and of equal importance from the theoretical point of view. It has been and continues to be a particularly difficult matter since neither the physiological nor immunological methods have been satisfactory. As a consequence there is basic disagreement among workers in this field as to what constitutes a species or variety and on what basis or bases differentiation should be made. Three general criteria have been used, *viz.*, hemolysis on blood agar plate culture, biochemical properties and immunological character as indicated by precipitin and agglutination reactions. Those concerned with the pathogenic streptococci

[11] Johnson and Hartman: Jour. Clin. Invest., 1947, 26:325.
[12] See Sherman: Bact. Rev., 1937, 1:1; Jour. Bact., 1938, 35:81.

make a tentative preliminary separation on the basis of hemolysis, and define species and types on an immunological basis. Workers with more general interests tend to rely primarily on physiological characters, and this is the basis of the Bergey (1948) classification.

Hemolysis. The use of blood plate hemolysis was introduced by Schottmüller in 1903 and is especially convenient since blood agar is the medium of choice in primary isolation. On this basis three types of streptococci may be distinguished:

(1) The β hemolytic streptococci which produce a clear zone of hemolysis in the red, opaque medium immediately surrounding the colony.

(2) The α hemolytic or green streptococci, which produce a zone of greenish discoloration in the medium about the colony which is considerably smaller than the clear zone of β hemolysis.

(3) The anhemolytic, indifferent, or γ streptococci which produce no change in the medium.
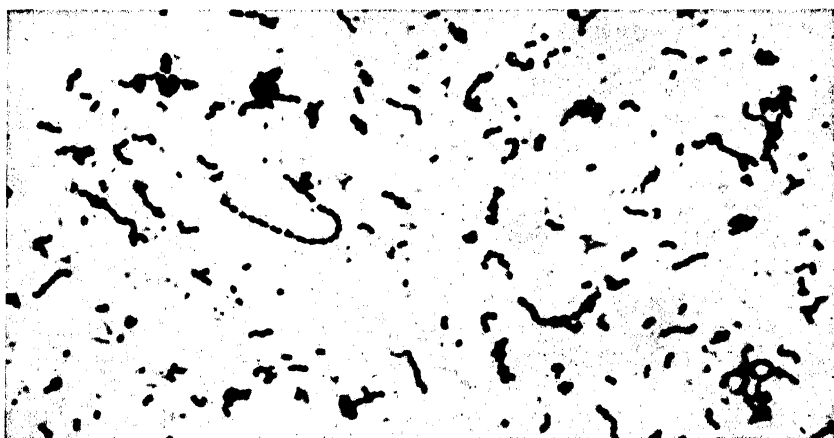
Fig. 48. *Streptococcus fecalis.* P strain isolated from focal infection. Smear from pure culture. Fuchsin; × 1050.

These distinctions have some validity in that the highly virulent streptococci isolated from pathologic conditions are almost invariably the β hemolytic variety. In the older literature these are grouped as a single species with the name *Streptococcus hemolyticus,* and some workers further differentiated on the basis of the disease with which the strain was associated, *viz., Streptococcus scarlatinae* (scarlet fever), *Streptococcus epidemicus* (epidemic septic sore throat), *Streptococcus erysipelatis* (erysipelas), etc. It is now quite clear that these distinctions are invalid in that identical streptococci may cause more than one clinical disease, and that the same disease may be caused by immunologically distinct streptococci; that is to say, there appears to be no basis for the concept of disease-specific types of streptococci, so far as diseases of man are concerned. The β hemolytic streptococci associated with diseases of lower animals do, however, show a high degree of specificity in the case of *Streptococcus equi,* causing strangles in horses, and *Streptococcus agalactiae,* causing mastitis in cattle, but not in that of the organism now known as *Streptococcus*

zooepidemicus which causes a wide variety of suppurative diseases in animals, including mastitis in cattle. The β hemolytic streptococci of human disease are usually not found in lower animals, but may occasionally infect the udder of the cow, giving rise to milk-borne septic sore throat. Conversely, those of animal origin do not ordinarily infect man, though human infections with *Str. zooepidemicus*, while not common, are somewhat more so than is generally believed. An even greater heterogeneity in the group is indicated by the fact that some non-pathogenic forms are also β hemolytic. It will be quite clear, therefore, that the inclusion of all these forms under the single species *Str. hemolyticus* is hardly justified.

A somewhat similar situation holds true with the α hemolytic or green streptococci which have been grouped as a single species, *Streptococcus viridans*. The green-producing group embraces such forms as the fecal strepto-cocci or enterococcus group, those which normally inhabit the mouth and throat, and non-pathogenic forms. Some of the α hemolytic forms, especially those found in the throat and intestine, are able to set up disease processes if normal resistance is reduced, with the production of localized infections at the roots of teeth, in the heart valves in bacterial endocarditis, etc. These patho-genic forms differ rather sharply from the highly virulent β hemolytic strepto-cocci, but again the entire group is too heterogeneous to justify inclusion in a single species.

The anhemolytic or indifferent streptococci are almost all saprophytic forms found in milk and various dairy products. The only pathologic condition with which they have been unequivocally associated is subacute bacterial endo-carditis,[13] in which they have been found in a small minority of cases. Here a variety of physiologically different types are included in a single group, and, as in the case of the other groups separated on the basis of hemolysis, it is too heterogeneous to allow lumping into the single species, *Streptococcus an-hemolyticus*.

Immunological Differentiation. Largely through the work of Lancefield and her co-workers the pattern of antigenic structure of the β hemolytic strepto-cocci has been defined, but the viridans and anhemolytic forms are serologically diverse so that this approach has not been useful. By the extraction of soluble antigens and application of the precipitin test, Lancefield[14] has demonstrated the presence of both group-specific and type-specific antigens in the hemolytic streptococci. The group-specific antigen, or "C" substance, is polysaccharide in nature and an integral part of the bacterial cell rather than a capsular material. On the basis of the specificity of this antigen five groups were originally de-scribed and designated Group A, Group B, and so on; additional groups have since been found up to and including Group N. The biological significance of these groups is indicated by the origin of the strains making them up, *viz.*:

Group A—Primarily pathogens of man
Group B—Found almost entirely in mastitis of cattle
Group C—Primarily pathogens of lower animals
Group D—Found in cheese
Group E—Found in milk

[13] See the discussion of this group by Rosebury: Medicine, 1944, 23:249.
[14] Jour. Exp. Med., 1925, 42:377, 397; *ibid.*, 1928, 47:91, 469, 481, 483, 857; *ibid.*, 1933, 57:571.

The association of origin and immunologic group is, however, not absolute. Streptococci of Group A are occasionally found in lower animals, producing mastitis in cattle for example, while those of Group C are found in man with some frequency as indicated in the accompanying table.

INCIDENCE OF STREPTOCOCCI OF GROUPS A AND C IN HUMAN INFECTIONS*

Type of Infection	Number of Strains	Group A		Group C	
		Number	Per cent	Number	Per cent
Scarlet fever..................	232	229	98.7	3	1.3
Tonsillitis and septic sore throat.....	52	52	100.0	0	0
Rheumatic fever and rheumatic arthritis........................	19	19	100.0	0	0
Puerperal sepsis..................	55	51	92.7	4	7.3
Erysipelas......................	51	48	94.1	3	5.9
Miscellaneous...................	143	136	95.1	7	4.9
Total.........................	552	535	96.9	17	3.1

* From Evans: Jour. Bact., 1944, 48:267.

Type specificity among the streptococci was indicated by the work of Griffith[15] who separated β hemolytic streptococci isolated from human disease into 27 types by slide agglutination, and later added three more types. These were given arbitrary arabic numbers, viz., Type 1, Type 2, etc. Following definition of the serological groups of Lancefield, it was found that these types are distributed over the groups; Group A included the majority, 23 in all, and Types 7, 20 and 21 fell into Group C, and Type 16 into Group D.

Lancefield has shown that the types within Group A are determined by two type-specific antigens. One of these, the "M" antigen, is a nucleoprotein which is destroyed by proteolytic enzymes, and may in fact be digested by a soluble protease produced by streptococci,[16] resulting in strains which cannot be typed by "M" antisera. The other type-specific antigen, designated the "T" antigen, is not as well known biochemically; it is resistant to proteolytic enzymes, but more recent work[17] suggests that it is also protein in nature. The "M" and "T" antigens are independent and may occur in various combinations. Thus, Types 10 and 12 contain the same "M" antigen but different "T" antigens, while Types 15, 17, 19, 23 and 30 contain very closely related "T" antigens but distinct "M" antigens. In addition, strains are found which lack

15 See Griffith: Jour. Hyg., 1935, 35:23.
16 Elliott: Jour. Exp. Med., 1945, 81:573.
17 Lancefield and Dole: Jour. Exp. Med., 1946, 84:449.

"T" antigen, and still others lack, or apparently lack, "M" antigen. While, then, the separation of the streptococci of Group A is clearly a complex matter, streptococcus typing has been rather generally applied, in part because it is of importance in the interrelationships, possibly phylogenetic, of the streptococci, and in part because it is of great value in epidemiological studies. Many workers feel that slide agglutination is not completely satisfactory and favor the precipitin reaction[18]; a method of carrying out the precipitin test in capillary tubes which is reliable and conserves serum has been developed. A detailed discussion of typing methods is given by Stewart, Lancefield, Wilson and Swift.[19]

In general these types are regarded as quite stable, but Evans[20] is of the opinion that they are not completely so and has reported that shifts in type occur on storage of cultures and as a result of animal passage. In this connection, Wilson[21] has found that a strain of Type 27 lost its group specificity, the "C" substance, during mouse passage, though type-specific precipitinogens were retained.

STREPTOCOCCAL ANTIGENS*

Group Specificity		Type Specificity		
Group	Antigen	Number of Types	Antigens	
			Name	Nature
A	"C" substance (polysaccharide)	More than 30	"M" "T"	Nucleoprotein Probably protein
B	"C" substance (polysaccharide)	4 main types plus subtypes	"S"	Polysaccharide
C	"C" substance (polysaccharide)	Possibly 13	No symbol	Protein
D et seq.	"C" substances (polysaccharides)	Several	"S"	Polysaccharides

* Modified from Lancefield: Harvey Lectures, 1940–41, Ser. 36, pp. 251–290.

Streptococci falling into other Lancefield groups on the basis of the specificity of the "C" substance are also divisible into types. In Group B, the bovine mastitis streptococci, four main types and a number of subtypes have been differentiated. In this group there is but a single type-specific antigen, and it is polysaccharide in nature. Group C contains, in addition to the three Griffith types noted above, ten additional types, five in strains of human origin and

[18] See Hilles and Hamburger: Jour. Inf. Dis., 1944, 75:265.
[19] Stewart, Lancefield, Wilson and Swift: Jour. Exp. Med., 1944, 79:99.
[20] Evans: Jour. Inf. Dis., 1946, 78:204.
[21] Wilson: Jour. Exp. Med., 1945, 81:593.

five in strains from horses, making thirteen in all. Here also there is but a single type-specific antigen and it is protein in nature. Types have been differentiated in several of the other groups on the basis of a single type-specific antigen, polysaccharide in nature.[22]

While the antigenic structure just discussed is largely that of the β hemolytic streptococci, β hemolysis is not invariably associated with the immunological character of these groups, and it has been found that certain of the enterococci, for example, contain these antigens. By and large, however, this antigenic structure does not extend far beyond the β hemolytic group and, as indicated above, immunological methods have not been useful in other than this group.

Physiological Differentiation. There is some correlation between the immunological groups of the hemolytic streptococci and their physiological characteristics. Group A shows a reasonable degree of biochemical homogeneity. All produce β hemolysis on blood agar and form soluble hemolysins, do not hydrolyze sodium hippurate, ferment trehalose but not sorbitol, do not reduce methylene blue, and seldom grow in 40 per cent bile agar. Group B is also more or less homogeneous, differing from Group A in that sodium hippurate is hydrolyzed and growth occurs in 40 per cent bile agar, but resembling that group with respect to methylene blue reduction and trehalose and sorbitol fermentations. The streptococci of Group C are considerably more diverse, and resemble Group A in that sodium hippurate is not hydrolyzed. Within the group there is some correlation between habitat and physiological character. Thus the strains isolated from strangles in horses are sorbitol, trehalose and lactose negative, while strains that are pathogenic for man generally ferment sorbitol but not trehalose.

Physiological characteristics have been used exclusively in the Bergey (1948) classification of the streptococci and the species differentiated on that basis are indicated in the following abridged key:

I. Facultative anaerobes
  A. Pyogenic group
    1. Sodium hippurate not hydrolyzed
      a. Lactose fermented
        i. Sorbitol −, trehalose +   **Lancefield Group A**
            Streptococcus pyogenes
        ii. Sorbitol +, trehalose −   **Lancefield Group C**
            Streptococcus zooepidemicus (animal pyogenes)
      b. Lactose fermentation variable
        i. Trehalose −
            Streptococcus equi
        ii. Trehalose +
            Streptococcus equisimilis
    2. Sodium hippurate hydrolyzed   **Lancefield Group B**
        Streptococcus agalactiae (*mastitidis*)
  B. Viridans group
    1. Lactose fermented
      a. Does not grow at 50° C.
        i. Starch not hydrolyzed, not tolerant of bile

[22] The biochemistry of the streptococcal antigens has been reviewed by Kabat: Jour. Immunol., 1943, 47:513; and by Lancefield: Jour. Exp. Med., 1943, 78:465; *ibid.*, 1944, 79:79.

Streptococcus salivarius*
Streptococcus mitis
 ii. Starch is hydrolyzed, bile tolerant
Streptococcus bovis
 b. Grows at 50° C.
Streptococcus thermophilus
 2. Lactose not fermented
Streptococcus equinus
C. Lactic group
 1. Maltose +, dextrin +, ammonia from peptone
Streptococcus lactis
 2. Maltose —, dextrin — (usually), no ammonia from peptone
Streptococcus cremoris
D. Enterococcus group   **Lancefield Group D**
 1. Not β hemolytic
  a. Does not hydrolyze gelatin
Streptococcus fecalis
  b. Gelatin hydrolyzed
Streptococcus liquefaciens
 2. β hemolytic
  a. Mannitol +, sorbitol +
Streptococcus zymogenes
  b. Mannitol —, sorbitol —
Streptococcus durans
II. Microaerophilic or obligate anaerobes
Streptococcus anaerobius
Streptococcus foetidus
Streptococcus putridus
Streptococcus lanceolatus
Streptococcus micros
Streptococcus parvulus
Streptococcus intermedius
Streptococcus evolutus

Most of these species are generally accepted and it will be clear from the foregoing discussion that all members of Group A are known as *Str. pyogenes,* the immunological varieties within the group being types of *Str. pyogenes.* Two species are included in Group C. The name *Str. zooepidemicus* is a new one introduced in the Bergey (1948) classification; it does not replace another name but rather gives species status to the animal pathogens of this group formerly casually known as "animal pyogenes" which rarely if ever occur in man. The other species, *Str. equisimilis,* includes those streptococci formerly known as "human C" and which are uncommon in lower animals. Of the β hemolytic streptococci found in man, 95 per cent or more are Group A and therefore *Str. pyogenes,* and the remainder, Group C, are *Str. equisimilis,* differentiation being made by the precipitin test, using group-specific antisera. Of the green streptococci found in man, the most common are *Str. fecalis* of the enterococcus group and *Str. mitis* and *Str. salivarius* of the viridans group. So far as the etiology of streptococcal infections in man is concerned this differentiation, and that of *Str. fecalis* and *Str. liquefaciens,* is unimportant; in fact it is open to serious question as to whether the last should be regarded as other than a variant of *Str. fecalis.* The identification of streptococci which do not fall into

* May be confused with the pneumococcus because it ferments inulin, but is distinguished by its lack of bile solubility.

the Lancefield groups is, of course, a matter of detailed biochemical study the details of which may be found in Bergey (1948).

Pathogenicity for Animals. As indicated above, certain of the streptococci are responsible for specific diseases of domestic animals. *Str. equi* is the cause of strangles in horses, a suppurative infection of the upper respiratory tract that is characterized by abscess formation in the throat and submaxillary region. This species is apparently not pathogenic for man. Other strains of Group C infect horses, causing respiratory catarrh and suppurative lesions in various parts of the body. The most common cause of streptococcal mastitis in the cow is *Str. agalactiae* which produces a chronic infection that is, as a rule, more common in older cattle. The infection is probably spread by the hands of the milker for the most part. Mastitis may also be caused by *Str. pyogenes* and may result in the spread of milk-borne streptococcal infection of man; it is probable that the source of infection is man and that *Str. pyogenes* does not occur naturally in the cow. Various other suppurative conditions, often involving infection of lymphatic tissue in animals such as the dog, sheep, etc., are of streptococcal etiology. Usually these are β hemolytic streptococci of groups other than Group A, but there are a few reports of spontaneous infections of lower animals with green streptococci.

Str. pyogenes is pathogenic for most laboratory animals, including the mouse, guinea pig and rabbit, but different strains vary widely in virulence for different animals; virulence may, of course, be enhanced by animal passage. Intravenous inoculation of virulent strains results in a fatal septicemia, suppurative peritonitis developing into septicemia follows intraperitoneal inoculation, and subcutaneous inoculation produces an abscess from which the infection may or may not spread. *Str. agalactiae* is of low virulence for experimental animals, and *Str. equi* is highly virulent only for the mouse. The green streptococci are also relatively avirulent for laboratory animals but may produce local infections on intravenous inoculation following injury; such techniques have been used to produce experimental arthritis lesions in the rabbit.

Pathogenicity for Man. The streptococci are responsible for a wide variety of diseases of man, perhaps a greater variety than any other kind of bacteria and, in addition to being the primary cause of disease, they have a marked tendency to occur in mixed and secondary infections with other pathogenic bacteria. In general the streptococcal infections are characterized by suppurative lesions and very often manifestations of toxemia, the latter taking the form of the so-called non-suppurative complications of the infection and including fever, arthritis, carditis and nephritis. They vary in the extent to which the body is involved, from local infections such as abscesses of the various tissues, including mucous membranes, joints and serous membranes, infection of the muscle or cellulitis which simulates gaseous gangrene, suppurative processes in all kinds of wounds, etc., to those generalizing to pyemia or septicemia. Some streptococcal infections have no distinctive clinical manifestations; thus an abscess caused by streptococci is not distinguishable from one of staphylococcal etiology. Others, however, such as erysipelas, streptococcus sore throat, scarlet fever, and the like have to a greater or lesser degree some distinctive clinical character.

The β hemolytic streptococci are by far the most virulent and, as indi-

cated above, the great majority of those infecting man are of Group A, *Str. pyogenes*. The small proportion of infections with Group C streptococci are not distinguishable from those of Group A except by isolation and immunological typing of the etiologic agent. The pathogenicity of these bacteria is accounted for to a considerable degree by the soluble toxic substances they produce. The most clear-cut example is that of the relation of the erythrogenic toxin to scarlet fever, but it is highly probable that other toxic substances also play a part in the development of the pathologic condition.

It seems plausible that the pronounced invasive tendencies of the streptococci are due in part to the elaboration of hyaluronidase by those varieties that are not encapsulated with hyaluronic acid. The association of fibrinolysin with virulence seems definite, so much so that this activity has been termed invasin by some workers. The role of the streptolysins in the development of the pathology of streptococcus infections is not too clear. Both streptolysin S and streptolysin O are toxic for experimental animals, the former causing death through intravascular hemolysis. The mechanism of the lethal action of streptolysin O is not known; it may possibly be related to the cardiotoxic action of this substance described by Bernheimer. There is some reason to believe that the destructive effect of streptococcal filtrates on polymorphonuclear leucocytes, attributed to the presence of a leucocidin, is closely related to or identical with streptolysin O, but in any case this activity may make up a significant part of the invasive and pathogenic properties of the streptococci. While the streptococci contain no endotoxin in the usual sense, it is of interest that the presence of the type-specific "M" antigen is associated with virulence, and antibody to it is protective while antibody to the type-specific "T" antigen is not.

In general, then, while knowledge of the mechanisms of the pathogenic action of the β hemolytic streptococci is far from complete, there is a considerable body of evidence which indicates that the production of disease is due at least in part to the action of the several toxic substances formed by these bacteria. As indicated earlier, strains of streptococci differ with respect to the kinds and amounts of such substances that they produce, and this variability accounts in part for the differences in disease they may produce. Thus, both strains which produce erythrogenic toxin and those which do not may produce septic sore throat, but only the former can produce scarlet fever also. These are not the only factors, of course, and the route of infection and immunity of the host are significant also; for example, wound infection and streptococcal sore throat involve different routes of infection, and an erythrogenic toxin-producing strain can produce sore throat but not scarlet fever in the immune.

Streptococci other than the β hemolytic varieties are much less virulent. The α hemolytic forms constitute, as pointed out elsewhere, a portion of the normal bacterial flora of the mouth, upper respiratory tract and intestinal tract. It is probable that they seldom initiate infection of the healthy tissues, but when natural resistance has been reduced they may be able to set up low grade, essentially localized infections such as focal abscesses in the teeth and gums. They are the most common cause of subacute bacterial endocarditis but, while the condition is a serious one, the infection shows little

or no tendency to spread throughout the body in spite of the frequent presence of the streptococci in the blood stream. Similarly, the α hemolytic streptococci are associated, probably causally, with rheumatic fever and arthritis, but the lesions are local and hypersensitivity to the streptococcal cell substance may be of considerable importance in the development of the disease. Of the anhemolytic streptococci, most are harmless saprophytes found in milk and dairy products, and these forms have been definitely associated only with subacute bacterial endocarditis, and then in a very small proportion of cases.

Epidemiology of Streptococcal Disease. The primary source of pathogenic streptococci is the human being who carries these bacteria in the upper respiratory tract. The infection may not be associated with symptoms and Group A and β hemolytic streptococcus carrier rates of 4 to 25 per cent have been reported by various workers. Those with overt symptoms of disease such as tonsillitis, pharyngitis, sinusitis and scarlet fever are, of course, prolific sources of infection. The importance of the carrier in the dissemination of streptococci has been studied extensively by Hamburger and his co-workers[23] who have shown that, while streptococci may be present in the saliva as well as in the throat, and discharged by sneezing, coughing and contamination of the hands, the nasal carrier is by far the most dangerous and contributes very large numbers of streptococci to his environment. Not only is the carrier the source of infection, but the fact that streptococcal disease may take a variety of clinical forms must be borne in mind. In an epidemic of scarlet fever, for example, the cases of pharyngitis and rhinitis are quite as important as those of frank scarlet fever, *i.e.*, those showing a rash, in the spread of the infection; in fact many workers record the incidence of scarlatinal rash in a given epidemic rather than differentiate scarlet fever from other streptococcal infection of the upper respiratory tract.

The transmission of streptococci from the infected person to the susceptible individual is in part a matter of direct contact and in part one of contamination of the environment, as Loosli and his co-workers[24] have pointed out. Direct contact may include inhalation of infective droplets expelled from the nose and mouth, hand to hand contact, etc., while contamination of the environment is the contamination of the air with droplets too small to settle, and through air and droplet infection the contamination of dust. Direct contact with the hands no doubt accounts for wound infection, puerperal fever, infection of the udder with *Str. pyogenes* to produce mastitis and milk-borne streptococcal disease, and possibly infection of the upper respiratory tract to a certain extent. Most upper respiratory tract infection is air-borne, either directly or through the agency of resuspended infected dust. The importance of the last is very great indeed (p. 231), and dust suppression measures such as oiling of blankets in hospital wards sharply reduce the incidence of streptococcal infection.

Immunity to Streptococcus Infection. Antibodies to the streptococcus cell substance and to the antigenic soluble products of these organisms are

[23] Hamburger *et al.*: Jour. Inf. Dis., 1944, 75:58, 71, 79; *ibid.*, 1945, 77:68, 96; Jour. Amer. Med. Assn., 1946, 130:836; Jour. Inf. Dis., 1946, 79:33.
[24] Loosli *et al.*: Jour. Inf. Dis., 1948, 82:59, 72.

formed following immunization or infection. In the case of *Str. pyogenes*, the former include the type-specific "M" and "T" antigens as well as the group-specific "C" substance. In the latter group are the erythrogenic toxin, streptolysins S and O, hyaluronidase and fibrinolysin. Antibody formation has no diagnostic utility in acute streptococcus infections, in part because there is not sufficient time for antibody formation during the course of the disease, at least in its early stages, and in part because isolation, and typing if desirable, of the infecting microorganism are relatively simple. Antibody titration is useful, however, for diagnosis in retrospect, so to speak, in associating streptococci with diseases such as rheumatic fever and arthritis, and for the determination of susceptibility to scarlet fever on the basis of antibody to the erythrogenic toxin.

The immunized animal responds to the cell antigens of streptococci with the production of precipitating and agglutinating antibody, and the use of such antisera makes possible the immunological grouping and typing of these bacteria discussed earlier. The immune response of man to these antigens during the course of infection appears more often to take the form of the development of a hypersensitivity, especially in the rheumatic diseases, and the immunological response may be measured by intradermal inoculation of soluble streptococcal antigens.[25] More often, however, serum is titrated for antibody to streptolysin or fibrolysin by *in vitro* methods. Quite aside from the question of the etiology of such diseases, it is clear that the occurrence of such antibodies in the human population is not uncommon, as might well be expected from the frequency with which streptococcal infection occurs, and it is clear that such infections do give rise to an antibody response. The erythrogenic toxin also stimulates the formation of specific antitoxin, following both clinical scarlet fever and immunization with the toxin, and, like diphtheria antitoxin, the incidence of its occurrence rises with age, indicating that immunization occurs without the intervention of clinical scarlet fever. Intradermal inoculation of erythrogenic toxin gives a skin test, analogous to the Schick test in diphtheria, which allows the estimation of the immunity of the individual to the toxin. This test, the Dick test, is discussed in the later section on scarlet fever. Antibody to the erythrogenic toxin cannot be titrated by *in vitro* methods, or to a satisfactory degree in experimental animals because of their relative lack of susceptibility to its action.

Differentiation must be made between an immune response in the technical sense of antibody formation and an effective immunity which prevents and/or modifies the infection or disease. The only immunity to streptococcal disease that is unquestionably effective is immunity to the erythrogenic toxin, which is reflected as immunity to the clinical disease scarlet fever. In general, however, immunity to streptococcus infections is of a low order and in any case transient. That some degree of effective immunity to a given type of streptococcus may be produced is indicated by recovery from and elimination of the infection in naturally occurring disease, and there is also some experimental evidence of the existence of an effective immunity. Wat-

[25] See, for example, Taran, Jablon and Weyr: Jour. Immunol., 1944, 49:209; *ibid.*, 1945, 51:53.

son, Rothbard and Swift,[26] for instance, have shown that a nasopharyngeal carrier state induced in monkeys resulted in an increase in antistreptolysin O titer of the serum and a resistance to reimplantation with the same strain that persisted for some months. Unfortunately such immunity appears to be type-specific and, as pointed out earlier, associated with antibody to the "M" antigen, and there is only a small degree of cross immunity. With the multiplicity of streptococcus types in the species *Str. pyogenes,* an effective immunity to streptococcus infection appears to be a somewhat impractical end.

Bacteriological Diagnosis of Streptococcal Infection. The isolation of streptococci from specimens of pathological material is ordinarily not difficult. An enriched medium is required and blood agar is the medium of choice, for both α hemolysis and β hemolysis are apparent. Overgrowth by Proteus in cultures of some kinds of specimens may be prevented by including 0.02 per cent sodium azide in the medium. Most specimens, such as throat swabs, pus, etc., may be streaked directly on blood agar but enrichment culture, as in veal infusion broth containing 0.1 per cent dextrose and 0.1 per cent phosphate buffer, should be made with blood taken for culture, incubated for twenty-four hours, and then streaked on blood agar. If there is reason to believe that the specimen contains sulfa drug, its bacteriostatic effect may be neutralized by including 5 mg. per 100 ml. of *p*-aminobenzoic acid in the medium.

The colonial morphology is typical in the case of β hemolytic streptococci and the characteristic chains of cocci may be found in gram-stained smears. Green streptococci from sputum and similar specimens must be differentiated from pneumococci by inulin fermentation and bile solubility. The hemolytic streptococci may be typed by agglutination with type-specific antisera and by the precipitin test. For the latter the sedimented bacteria from a 250 ml. broth culture are suspended in 10 ml. of N/10 HCl in saline, boiled for ten minutes, cooled in running water, and the insoluble material spun out to leave a clear supernatant to be used as the antigen. Considerable economy of reagents may be effected by setting up the precipitin test as a ring test in capillary pipettes; the precipitate at the serum-antigen interface may be observed with a hand lens.[27]

STREPTOCOCCAL INFECTION OF THE SKIN AND SUBCUTANEOUS TISSUES

Erysipelas. The ability of streptococci to infect the skin and adjacent tissues is well illustrated in erysipelas, an inflammatory disease of the skin caused by *Str. pyogenes.* There is some evidence that attack of the disease is preceded by streptococcal infection of the throat or elsewhere in the upper respiratory tract, and it has been found that some individuals at least have the same immunological type of streptococcus in the throat as in the skin lesions. It is not clear whether the skin is directly invaded, or whether the microorganisms reach the area by some internal route, but the latter is only suggested and by no means established. The etiologic relationship of *Str. pyogenes* to the disease is indicated by its presence, frequently in enormous

[26] Watson, Rothbard and Swift: Jour. Exp. Med., 1946, 84:127.
[27] Swift, Wilson and Lancefield: Jour. Exp. Med., 1943, 78:127.

numbers, in the lesions, the production of erysipelas-like disease in rabbits by the inoculation of streptococci, and inoculation experiments in carcinomatous patients that have demonstrated that pure cultures of streptococci can provoke the erysipelatous process.

Streptococci are not present in the central portion of the inflamed area, but are found on its periphery, and can be isolated most readily by excision of portions of the tissue, other methods rarely succeeding. In the skin they occur chiefly in the lymph spaces, which are often packed with them, and may be recovered by skin puncture as far as 3 cm. beyond the advancing edge of the lesion where there is no gross evidence of inflammation. The hypothesis that the inflammatory reaction is due in part at least to the erythrogenic toxin has been an attractive one, but it seems definitely established that there is no relation; immunization with erythrogenic toxin, for instance, in no way prevents or reduces the inflammatory reaction in erysipelas.

This disease, and especially experimental erysipelas in the rabbit, has been of very considerable interest in connection with problems of local and tissue immunity. It is well established that one attack of the disease confers no protection against subsequent attacks, and in the opinion of many, some persons have a predisposition to the disease, suffering repeated attacks throughout life and even in the same areas. In the experimental disease in the rabbit, however, a number of workers[28] have reported that successive intradermal inoculations bring about an increased resistance to subsequent inoculation in that area, and that with continued immunization the area increases slowly in size. The immunity is not highly specific and inoculation of sterile broth also results in some increase in resistance to infection. Consistent with this, immune serum, when mixed with streptococci and inoculated intradermally, seems to have some protective effect. Serum therapy of the human disease, however, appears to produce only a possible mildly favorable effect upon the immediate attack, but none upon recurrences and complications such as abscess formation.

Wound Infection. The green streptococci seldom occur in infections of wounds, but *Str. pyogenes* produces a suppurative infection when present, especially late. This organism is not normally present on the skin and, in fact, normal skin has a pronounced bactericidal effect upon it (p. 224). The relative rarity of infection of wounds by these bacteria is consistent with this, and streptococcal infection in most instances is a result of subsequent contamination by direct contact rather than of a primary infection. *Str. pyogenes* may occur alone or in mixed infections with other pyogenic bacteria such as staphylococci.

Traumatic invasion of the skin and subcutaneous tissues may not remain localized, but may develop into an acute, spreading infection of the subcutaneous tissue with invasion of the muscle giving rise to a gangrenous myositis. The infection of the subcutaneous tissues may show little or no evidence of localization and is characterized by the formation of a seropurulent exudate. It tends to spread rapidly via the lymphatic tissues and generalize into septicemia. This kind of streptococcal infection has been

[28] See, for instance, Amoss and Bliss: Jour. Exp. Med., 1927, 45:411.

termed *cellulitis,* and may result from *Str. pyogenes* alone or, in the development of the gangrenous process, more often in mixed infection with anaerobic streptococci. This kind of wound infection was found with some frequency in World War II.[29]

STREPTOCOCCAL INFECTION OF THE UPPER RESPIRATORY TRACT

As indicated earlier, the β hemolytic streptococci occur most commonly as parasites and pathogens of the upper respiratory tract. By far the largest proportion of human disease caused by *Str. pyogenes* results from infection of the upper respiratory tract and adjacent areas, symptoms arising not only from the acute infectious process, but also in connection with its complications. The clinical character of the disease is determined by the relative prominence of the various results of the infection and, while seemingly different, is fundamentally the same. Thus streptococcal pharyngitis or septic sore throat becomes scarlet fever when the infecting strain of *Str. pyogenes* produces erythrogenic toxin, the infection commonly extends into the tonsils or may be localized primarily there to give clinical tonsillitis, it may extend into the sinuses or middle ear to produce streptococcal sinusitis and otitis media respectively, and by extension into the lungs result in bronchopneumonia of streptococcal etiology. Furthermore, the so-called late nonsuppurative complications of streptococcal infection include carditis, nephritis and arthritis. It will be clear, then, that while separation of β hemolytic streptococcus infection into various clinical entities has some practical value, the basic infectious process is essentially the same.

The green streptococci are also inhabitants of the upper respiratory tract as pointed out earlier, and are so constantly present and of such restricted pathogenicity that they are a part of the normal bacterial flora. Infection of the teeth and gums undoubtedly stems from this area, and it is not unlikely· that the bacteria entering the blood stream to produce local infections elsewhere in the body originated in the upper respiratory tract. They are occasionally found in bronchopneumonia.

Streptococcus Sore Throat (Septic Sore Throat). The β hemolytic streptococci are responsible for an acute infection of the throat commonly known as septic or streptococcus sore throat. Epidemics of this disease appeared in the United States and in England during the first decade of the present century. The immediate symptoms in these and subsequent epidemics have been strikingly similar, and include an intense local hyperemia, with or without a grayish exudate, enlargement of the cervical lymph nodes, and usually fever. Extension of the infection into the lungs may occur with a resulting streptococcus pneumonia which may terminate in a fatal septicemia, and peritonitis has been a cause of death also. The disease, usually in a relatively mild form, is a relatively common one; 9197 cases and 511 deaths were reported in 1945 from 36 states, rates of 11.9 and 0.7 per 100,000 respectively, and many cases are, of course, not reported.

The sequelae of streptococcal sore throat include those resulting from the extension of the infection into adjacent areas such as the sinuses and middle ear, and purulent, semi-chronic infections often develop. Streptococci

[29] MacLennon: Lancet, 1943, *i:*582; *ibid., ii:*63, 94, 123.

also frequently persist in tonsillar crypts in a chronic type of infection which may flare up periodically in an acute form. Streptococcal tonsillitis, sinusitis and otitis media are, then, a part of the pathology of hemolytic streptococcus infection of the throat. In addition to such extensions, the effects of toxemia on other parts of the body are evident as carditis, nephritis and arthritic involvement of the joints. As in the case of scarlatinal rash noted earlier, the character of an epidemic is often recorded as percentage incidence of these various sequelae.

The distinctive clinical character and epidemic spread of the disease led earlier workers to believe that it was caused by a particular kind of streptococcus to which the name *Str. epidemicus* was given. It is now clear that various immunologic types of *Str. pyogenes* are, for the most part, responsible for the disease, and a small portion of the cases are infections with streptococci of Group C, now grouped as *Str. equisimilis*. On the other hand, there is reason to believe that so-called "epidemic strains," of high virulence and infectivity, of streptococci as well as other bacteria, are often associated with epidemic disease.

It is probable that the infection is largely droplet and air-borne, including dust, but there is no doubt that in many instances direct contact is of considerable significance. It may also be transmitted through the agency of food and milk, giving rise to epidemics among the consumers of these products. Hamburger, Green and Hamburger[30] have reported a food-borne epidemic of pharyngitis-tonsillitis among convalescent patients in which a food handler was found to be in the incubation period of pharyngitis-sinusitis caused by *Str. pyogenes* Type 1; his nose ran profusely, he had strongly positive nose and throat cultures, and 10,000,000 streptococci were recovered in a single culture of his hands. There is no doubt also that streptococcal infection is frequently milk-borne. In the past it has been assumed that the contamination was direct from man, but in recent years evidence has accumulated which indicates that mastitis of *Str. pyogenes* etiology may constitute the immediate source of infection of the milk.

SCARLET FEVER

Scarlet fever is a clinical entity because of the rash resulting from the action of the erythrogenic toxin; otherwise it does not differ significantly from other streptococcus infection of the upper respiratory tract and its sequelae are essentially the same. The relationship of β hemolytic streptococci to the disease was demonstrated by Dick and Dick in 1923[31] by the reproduction of typical scarlet fever in human volunteers by the inoculation of pure cultures, and by the demonstration of the existence of the erythrogenic toxin. A conclusive demonstration of the etiologic relation was required because of the contrast between the relatively lasting immunity to scarlet fever following recovery from an attack of the disease and the transient immunity to other streptococcal infections.

It has been held by the Dicks and others, largely on the basis of early

[30] Hamburger, Green and Hamburger: Jour. Inf. Dis., 1945, 77:96.
[31] For a general discussion see Dick and Dick: *Scarlet Fever*. Year Book Publishers, Chicago. 1938.

agglutination studies, that the scarlet fever streptococci constitute a homogeneous group of β hemolytic streptococci which should be designated *Str. scarlatinae.* While the streptococci found in scarlet fever are all members of Group A, *i.e., Str. pyogenes,* the ability to form erythrogenic toxin is not confined to any particular type within this group, though some types occur more frequently than others, and it cannot be said that the scarlet fever streptococci are immunologically homogeneous. Neither are they biochemically homogeneous, and in the Dicks' early experiments the mannitol fermentation was variable in scarlet fever-producing strains. It appears, then, that the scarlet fever streptococci are strains of *Str. pyogenes* that have in common
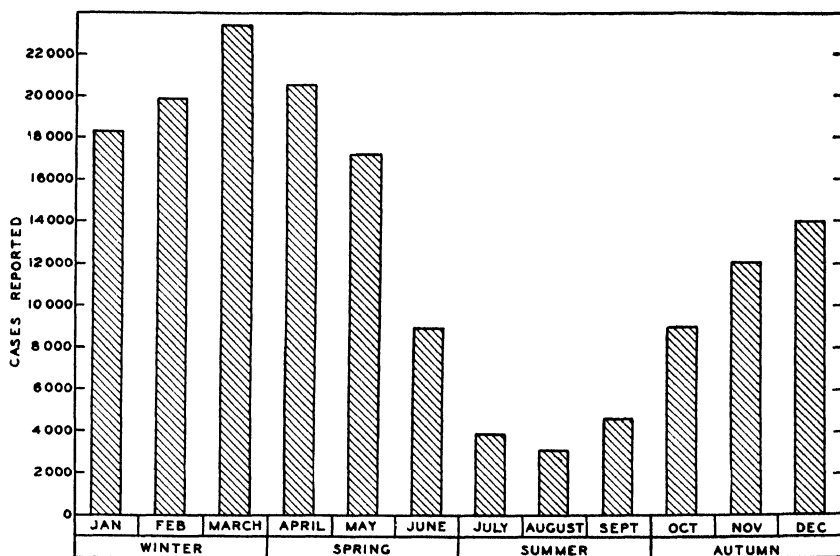
Fig. 49. The seasonal incidence of scarlet fever. Averages of reported cases by months for the years 1939 to 1945 inclusive. Data from Supplements to Public Health Reports.

the ability to form erythrogenic toxin, but such strains are found in conditions other than scarlet fever, such as erysipelas.

The Erythrogenic Toxin. The erythrogenic toxin differs from the other soluble bacterial toxins in that it is resistant to heat, as noted earlier. Its nature is not clear as yet, however. It has been reported to be a protein by some workers and a polysaccharide-containing complex by others.[32] Barron, Dick and Lyman[33] have reported that it is a protein of low molecular weight and is resistant to proteolytic digestion and a number of oxidizing and reducing agents. Stock[34] has prepared a highly purified heat-coagulable protein containing 10^7 STD per milligram which he regards as identical with the erythrogenic toxin. Four components have been demonstrated by electrophoresis; the toxicity was associated with the slowest and on separation this contained 10^8 STD per milligram.[35] Whatever its nature, it is necessarily

[32] Earlier work is reviewed by Eaton: Bact. Rev., 1938, 2:3.
[33] Barron, Dick and Lyman: Jour. Biol. Chem., 1941, 137:267.
[34] Stock: Jour. Biol. Chem., 1942, 142:777.
[35] Krejci, Stock, Sanigar and Kraemer: Jour. Biol. Chem., 1942, 142:785.

assayed by skin test and its potency is measured in skin test doses (STD), the smallest amount that will produce the characteristic erythematous response. It is, of course, antigenic, and is fluocculated by antitoxic sera.

Scarlet Fever Antitoxin. The therapeutic use of antistreptococcus sera was investigated by Moser in 1902 with encouraging results, and similar observations were reported by Dochez and others in 1924. The Dicks prepared specific antitoxic sera by immunizing horses with sterile culture filtrates. The results of the therapeutic use of the Dick antitoxin are favorable on the whole, though the mildness of the prevalent type of scarlet fever makes it difficult to secure any large body of statistics as cogent as those recorded in diphtheria. In individual cases, however, the administration of antitoxin decreases the duration of the rash, changes the character and extent of desquamation and reduces the number of complications, and there is general agreement as to the efficacy of antitoxic serum properly prepared and administered early. Some clinicians would restrict the use of scarlet fever antitoxin to cases of severe or toxemic type. A unit of antitoxin is defined as that amount which will neutralize 50 skin test doses of toxin.

Immunity to scarlet fever is, in a sense, a clinical immunity in that it is largely an immunity to the erythrogenic toxin rather than to the streptococcus. It may be demonstrated by the *Dick test,* a skin test analogous to the Schick test in diphtheria, *i.e.,* the local erythema is due to the action of the toxin and is absent in the presence of antitoxin, either of exogenous origin or present in the immune individual. The Dick test may be used, then, to ascertain whether or not an individual is immune to scarlet fever or, more precisely, whether circulating antitoxin is present. In this connection it is of interest that Schulz and Charlton[36] earlier observed that when a scarlet fever patient with a bright-red rash is injected with 1 ml. of convalescent serum, after about six hours the rash begins to fade and soon disappears completely. The significance of this phenomenon, the *Schultz-Charlton blanching phenomenon,* was not recognized at the time.

Prophylactic Inoculation. It is not generally known that following Jenner's work on smallpox vaccination (p. 6) attempts were made to immunize against scarlet fever by a similar process of inoculation. Preventive inoculation with killed streptococcus cultures was practiced by Russian bacteriologists as early as 1906. Mild symptoms were produced similar to those observed in scarlet fever. A single injection did not suffice to produce immunity, two or three inoculations being necessary. It was believed that a considerable degree of immunity was obtained by this procedure.

The discovery of the scarlet fever toxin offered an opportunity for protective immunization similar to that successfully utilized in diphtheria. Toxins with a potency of at least 40,000 STD per cubic centimeter are preferable, and appropriate dilutions are injected at intervals of one week. Five injections are recommended, starting with 500 STD and gradually rising to about 100,000 STD. Immunization of susceptibles (Dick positives) in this manner produces 98 per cent or more Dick negatives. The injections are sometimes accompanied by the development of a scarlatiniform rash and other symptoms of mild scarlet fever. In consequence, the use of toxin

[36] Schultz and Charlton: Ztschr. f. Kinderheilk., 1918, Orig. 17:328.

detoxified by treatment with formalin has been advocated by some workers. Veldee[37] has reported that a purified precipitated toxin apparently produces a good immunity with fewer untoward reactions. It is to be emphasized, however, that immunity to scarlet fever is an immunity to clinical scarlet fever, not to the streptococcal infection, and from the epidemiological point of view both Dick-negative and Dick-positive individuals must be considered as having scarlatinal infections, the only difference being the clinical one dependent upon the development of a rash.

As in the case of diphtheria, immunity to scarlet fever may be acquired by inapparent infection (p. 621). The frequency of positive Dick tests is low in newborn children (indicating a passive immunity of maternal origin), then rises to a maximum in the age groups one to five years, and thereafter
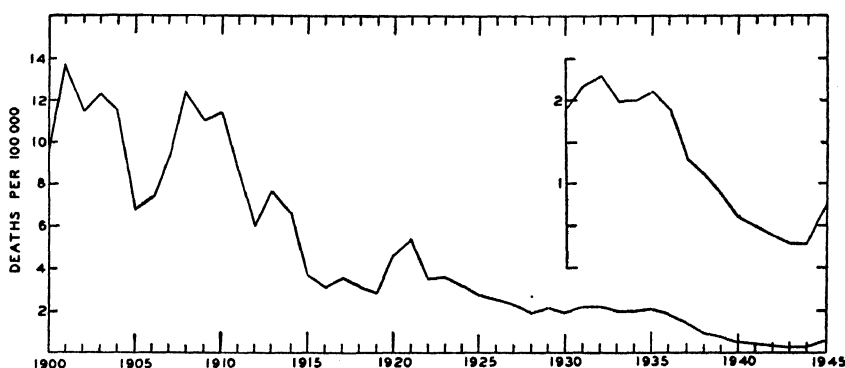
Fig. 50. The prevalence of scarlet fever in the Registration Area since 1900 as indicated by the uncorrected death rates. From data compiled by the Bureau of the Census.

falls off gradually until in persons over thirty it is relatively low, possibly 15 per cent.

RHEUMATIC FEVER

As indicated earlier, not uncommon sequelae of β hemolytic streptococcus infection include carditis and arthritic involvement of the joints. These are emphasized and assume a major role in the pathology and symptomatology of the post-streptococcal non-suppurative inflammatory disease known as rheumatic fever, acute rheumatism or rheumatic heart disease. The essential lesion of rheumatic fever is carditis which may or may not be accompanied by pyrexia and arthritis. The carditis includes the connective tissue degeneration characteristic of the damaged heart valves, and specific inflammatory myocardial lesions characterized histologically by nodular collections of cells described by Aschoff and known as Aschoff nodules.[38]

The connection between β hemolytic streptococcus infection and rheumatic fever is very close and there is good reason to believe, though as yet not complete and unequivocal proof, that the relationship is etiological. The epi-

[37] *Cf.* Veldee, Peck, Franklin and DuPay: Pub. Health Repts., 1941, 56:957.

[38] See the general reviews by Griffith: Jour. Amer. Med. Assn., 1947, 133:974; and Lancet, 1947, i:217.

demiological association of streptococcal infections such as tonsillitis, puerperal fever, scarlet fever and the like with rheumatic disease has long been recognized, and is reinforced by evidence of an immunological response, such as antistreptolysin O, to streptococcal infection almost invariably associated with rheumatism. The onset of the disease does not necessarily coincide with acute streptococcal disease, however, and only rarely has it been possible to culture β hemolytic streptococci from the blood. Largely through the work of Coburn[39] beginning in 1931 these discrepancies have been reconciled and the etiologic relationships of the β hemolytic streptococci clearly indicated.

Coburn differentiates three stages in the disease: first, an acute streptococcal infection of the nasopharynx; second, a period of quiescence during which, however, streptococci still remain in the throat; and third, the stage in which electrocardiographic changes are apparent and the symptoms of acute rheumatic fever appear. The period of time elapsing between the first and third stages varies from one to five weeks in patients in which it has been studied. Subsequent attacks of rheumatic fever occur on reinfection with streptococci or even as a consequence of non-specific events such as bone fracture or severe sunburn.

The mechanism by which streptococci produce rheumatic fever is far from clear. The antibody response to the initial infection is delayed in that peak titers of antistreptolysin O, complement-fixing antibody and group and type-specific precipitins occur in the third stage of the disease rather than earlier, while antistreptolysin S declines in the third stage, particularly in those having recrudescences. There is reason to believe that hypersensitivity exists to the streptococcus or its products; for instance joint pains are produced in rheumatic patients with a high degree of frequency by the inoculation of sterile streptococcus culture filtrates. In any case, the immunological response, whether clearcut antibody formation, hypersensitivity, or the purely hypothetical suggestion of Coburn that a haptene of human origin forms an antigenic complex with streptococcal protein, appears to be intimately associated with the development of the disease. Whatever antigen may be concerned is widely distributed among strains of *Str. pyogenes*, for various immunologic types are associated with the disease.

It is quite likely also that the role of the host is of considerable importance. There is some indication of a constitutional predisposition to rheumatic fever; the disease occurs more commonly in hyperthyroid persons, there is some evidence suggesting that lipid metabolism may be involved, and it occurs more commonly in the five to fifteen years age group than in older persons.

It has been of some interest that many of the lesions characteristic of rheumatic fever may be produced in the rabbit by inoculation of β hemolytic streptococci. These include non-suppurative myocarditis (without, however, the typical Aschoff nodule), endocarditis, and arthritis ranging from mild to severe with extensive changes in the bone. While it may be argued that such pathology is not identical in all respects with that of rheumatic fever in man, the fact that such lesions can be produced experimentally by the inoculation of streptococci may be regarded as strong evidence in support of the assumption that these bacteria may be etiologically related to human rheumatic fever.

[39] See the general discussion by Coburn: Amer. Jour. Dis. Children, 1945, 70:339, 348.

In general, then, it seems quite clear that there is a very close, probably etiological, relation between *Str. pyogenes* and rheumatic fever, but the pathogenesis of the disease is far from clear.

SUBACUTE BACTERIAL ENDOCARDITIS

Infection of the endocardium with the formation of ulcerative lesions may occur with many bacteria. By far the most common are the α hemolytic streptococci but staphylococci, β hemolytic streptococci, and to a much lesser exent, pneumococci, gonococci, meningococci, Hemophilus, Brucella, Salmonella, etc., are found. With the more highly virulent bacteria the infection is an acute one, but the disease known as subacute bacterial endocarditis, which is no less fatal, is caused almost exclusively by α hemolytic streptococci, though in a very few instances anhemolytic streptococci have been implicated.

The infection of the heart valves may be primary or secondary to a focus of infection elsewhere in the body, and an almost indispensable predisposing factor is congenital abnormality or prior damage, as by rheumatic infection, of the heart valves. The source of infection is often the tonsils and periapical infections of the teeth or low grade infection in the gums. There seems to be an occasional leakage of streptococci from these sources into the blood stream, and any disturbance of the infected areas, such as the extraction of teeth, tonsillectomy, manipulation of an infected cervix, etc., results in a transient bacteremia.[40] The bacteria are rapidly removed from the blood stream by phagocytic cells in the liver, spleen, bone marrow and elsewhere in the body, but infection of the heart valves may occur when there is an abnormality.

The infection of the endocardium is probably direct rather than from embolism in the smaller vessels at the attachment of the endocardial and subendocardial tissues. According to Grant, Wood and Jones[41] the bacteria establish in small platelet thrombi on the valvular surfaces, but McNeal, Spence and Slavkin[42] found that, in experimental endocarditis in the rabbit, the circulating bacteria are phagocytosed by the endothelial cells on the heart valves as well as elsewhere, but are not killed and produce local damage which is covered by a deposit of fibrin; the fibrin protects the streptococci to allow proliferation and the development of a local infection. In any case, during the course of the disease streptococci are shed into the bloodstream and may be demonstrated by blood culture.

Species of green streptococci of both the viridans and enterococcus groups are responsible for this disease. Hucker[43] found that practically all of nearly 200 cultures of green streptococci from clinical sources, including subacute bacterial endocarditis, were either *Str. salivarius* or *Str. fecalis,* and Willius[44] has reported that the most common species found is *Str. mitis, Str. salivarius* is next, and *Str. fecalis* is found least often. The disease process is, however, essentially the same regardless of the species of α hemolytic streptococcus causing it.

[40] See Okell and Elliott: Lancet, 1935, *ii:*869; Elliott: *ibid.,* 1939, *ii:*589.
[41] Grant, Wood and Jones: Heart, 1928, *14:*247.
[42] McNeal, Spence and Slavkin: Amer. Jour. Path., 1944, 20:5.
[43] Quoted by Sherman: Bact. Rev., 1937, *1:*1.
[44] Willius: Proc. Staff Meet. Mayo Clinic, 1944, 19:380.

14

PUERPERAL FEVER

Puerperal fever is a vague term in that some degree of pyrexia is not uncommon immediately following childbirth. A definite febrile response is probably in most instances associated with bacterial infection but in mild cases the microorganisms are relatively avirulent. The severe infections, however, are due almost entirely to streptococci, the majority *Str. pyogenes*. Infection with other β hemolytic streptococci occurs but when these are of groups other than Group A the infections are usually not severe. The anaerobic streptococci are second only to *Str. pyogenes* in importance, being the cause of possibly 20 to 25 per cent of cases of severe puerperal fever; the disease caused by these forms is not as rapidly fulminating as that caused by *Str. pyogenes*, but the case fatality rate is high, possibly 40 per cent. In fatal cases of puerperal fever the infection generalizes to a fatal septicemia and it is highly probable that the pronounced invasive qualities of the streptococci are responsible for their virulence under these circumstances.

A point that has been of primary interest is the source of the streptococci producing puerperal fever. It seems well established that Str. *pyogenes* occurs only rarely in the female genital tract and is rarely found before labor or during an afebrile puerperium. Lancefield and Hare,[45] for example, reported a series of 855 cultures of the vagina of parturient women; one strain of *Str. pyogenes* was found among 65 strains isolated during an afebrile puerperium, and none among 13 strains isolated before labor. This and similar studies indicate that the source of infection with *Str. pyogenes* is exogenous rather than endogenous. The development of streptococcus typing and a reasonably precise definition of the immunological types has allowed the demonstration of the probable source of the infecting organisms. In the studies reported by Smith[46] slightly over 50 per cent of the streptococci from the patient were identical with those from the nose and throat of attendants, and a little less than 25 per cent identical with those from the nose and throat of the patient. Colebrook[47] reported similar results, the percentages being 58 and 38 respectively. Data such as these show clearly that the source of infection is exogenous with respect to the genital tract and most probably the nose and throat of attending persons or the patient herself in 75 per cent or more of cases. The infection may be air- or dust-borne, but it seems probable that the hands play a significant part in the transfer of infection.

The anaerobic streptococci, on the other hand, are normal inhabitants of the human vagina. In the absence of precise immunological methods for their identification, it may be tentatively concluded that infection with these organisms to produce puerperal fever is probably endogenous for the most part.

[45] Lancefield and Hare: Jour. Exp. Med., 1935, *61*:335.
[46] Smith: *Causation and Source of Infection in Puerperal Fever.* H.M. Stationery Office, London. 1931.
[47] Colebrook: Med. Res. Council, Spec. Rept. Series No. 205, 1935.

THE PNEUMOCOCCI

The bacterium most commonly found in pneumonia in man is a small lanceolate micrococcus which has been variously termed *Micrococcus pneumoniae, Micrococcus lanceolatus, Streptococcus pneumoniae,* or more briefly, the *pneumococcus* or *Fränkel's pneumococcus. Diplococcus pneumoniae* is now the commonly accepted formal name.

THE INCIDENCE OF ETIOLOGIC AGENTS IN PNEUMONIA*

Causative Organism	Lobar Pneumonia	Broncho-pneumonia	Unspecified	All Pneu-monias
Pneumococcus................	82.48	65.79	77.48	77.71
Hemolytic streptococcus......	2.00	3.33	3.99	2.65
Other streptococci..........	1.30	2.99	1.33	1.70
Staphylococcus..............	.82	2.00	1.38	1.19
Friedländer's bacillus........	.15	.13	.28	.17
Influenza bacillus............	.06	.25	.11	.15
Tubercle bacillus............	.....	.08	.....	.02
Fungi......................	.....	.02	.....	.....
Virus......................	.....	.....	.07	.01
No significant organism recorded..................	13.19	25.41	15.38	16.44
Number of cases............	15,420	6,092	4,290	25,802

* In six representative states over a two-year period as compiled by Rumreich *et al.*: Pub. Health Repts., 1943, *58*:121.

Of the generally recognized anatomical types of pneumonia, lobar or acute croupous pneumonia, bronchopneumonia or lobular pneumonia, and capillary bronchitis or bronchiolitis, lobar pneumonia is nearly always due to the pneumococcus, though other bacteria are occasionally involved. Perhaps the best quantitative data are those assembled by Rumreich and his co-workers[1] in a two-year study in six states representing high and low pneumonia rates. These are summarized in the accompanying table. It will be clear that the pneumococcus is by far the commonest cause of pneumonia. The microorganisms associated with bronchopneumonia are varied (see table) and their source is probably almost always the nasopharynx.[2] Pneumonia is a relatively common disease; 101,811 cases and 34,421 deaths were reported in 1945 by 31 states, rates of 124.3 and 42.0 per 100,000 population respectively.

[1] Rumreich *et al.*: Pub. Health Repts., 1943, 58:121.
[2] See Smillie and Duerschner: Amer. Jour. Hyg., 1947, 45:1, 13.

The pneumococcus was discovered independently in France in 1881 by Pasteur, who inoculated rabbits with the saliva of a child dead of rabies, and by Sternberg in the United States, but was not known to be associated with disease in man before the extensive investigations of Fränkel and Weichselbaum, who demonstrated conclusively the etiological relation of this bacterium to pneumonia in man.

Morphology and Staining.[3] The pneumococcus is typically a small, slightly elongated coccus, one end of which is pointed or lance-shaped. The cocci commonly occur in pairs (diplococci), but variations both in grouping and in size and form of individual cells are frequently observed. Chain formation is common, especially in artificial media, although the chains are usually shorter than those of *Streptococcus pyogenes.* Oval and elongated bacillary forms sometimes occur. The pneumococcus is non-motile and does not form spores. A well-defined capsule envelops the pneumococci in animal
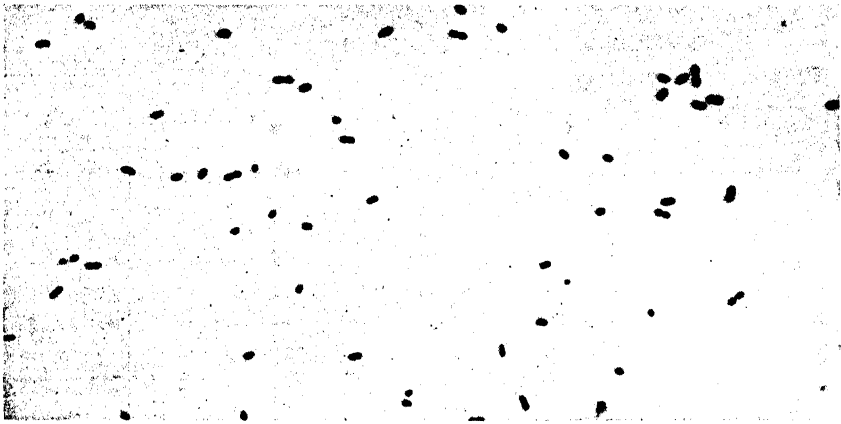
Fig. 51. Pneumococcus, pure culture. Note the typical lanceolate shape and diplococcus arrangement. Fuchsin; × 1050.

exudates but, except in certain strains or in certain media, is less readily demonstrable in cultures grown outside the body. Capsules may often be found in milk cultures and in media containing blood or serum.

The pneumococcus is readily stained with the aniline dyes and is generally gram-positive, although there is a tendency to become gram-negative in older cultures and occasional strains are found to be gram-negative. In stained preparations the capsule may often be seen as an unstained halo surrounding the cells; it may be stained by special methods.

The colonies of pneumococcus on infusion agar or blood agar are typically small, moist, translucent and granular, with well-defined edges. These bacteria are α hemolytic on blood agar, and the colonies appear surrounded by a zone of greenish discoloration on this medium and resemble the colonies of green-producing streptococci.

Physiology. Some pneumococci grow upon the ordinary nutrient (beef extract) culture media but many do not, and in any case growth is sparse. Nu-

[3] The pneumococcus is completely and exhaustively discussed by White: *The Biology of Pneumococcus.* The Commonwealth Fund, New York. 1938.

tritional requirements are complex; Rane and Subbarow[4] have been able to grow Types 1, 2, 5 and 8 but not Type 7 in a medium consisting of acid hydrolysate of gelatin supplemented with glutamic acid, cystine, glucose, pantothenic acid, nicotinic acid, choline and thioglycollic acid. Similar observations have been reported by other workers.[5] Growth on infusion media, particularly those enriched by the addition of whole blood, takes place at 37° C. Litmus milk is promptly acidified and often, but not invariably, coagulated. The temperature range over which these bacteria may be grown is relatively narrow (25° to 42° C.), and they are sensitive to variations in pH from the optimum of 7.8, the limiting acidity and alkalinity being 7.0 and 8.3 respectively. The pneumococcus is a facultative anaerobe, although certain other species of Diplococcus are obligate anaerobes.

In general, sugars are actively fermented with the production of large amounts of lactic acid and small amounts of volatile acid and ethyl alcohol.
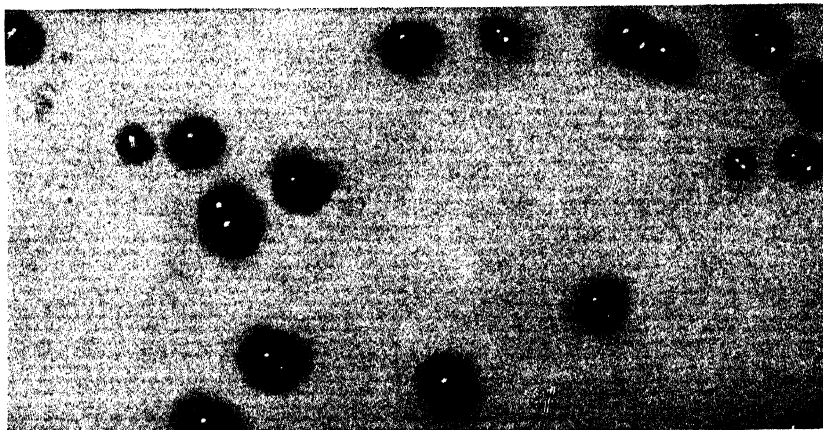
Fig. 52. Colonies of the pneumococcus on blood agar. The areas of green hemolysis have been accentuated in the photograph. × 3.

Differential fermentations are of no particular value in the classification of these microorganisms except in the case of inulin, which serves to differentiate the pneumococci and the green streptococci.

The pneumococci appear to be structurally delicate organisms and autolyze much more readily than most other kinds of bacteria. Evidences of protein hydrolysis accompany autolysis, and it appears that lysis is a consequence of the activity of intracellular ferments. Perhaps associated with the autolytic process is the lysis of pneumococci by bile and bile salts. The so-called "bile solubility" of pneumococci is practically a constant characteristic, although different strains vary in their sensitivity to bile as they do in the tendency to autolysis. Rabbit or ox bile may be used and added to a young broth culture in the proportion of 10 to 20 per cent. Solutions of pure bile salt (sodium taurocholate) are preferable to ox bile because they may be sterilized and the concentration

[4] Rane and Subbarow: Jour. Bact., 1940, 40:695.

[5] Gilbert: Proc. Soc. Exp. Biol. Med., 1944, 57:363; Adams and Roe: Jour. Bact., 1945, 49:401.

(10 per cent) controlled. Heat-killed pneumococci are not bile-soluble. Sodium lauryl sulfate and similar detergents will also lyse pneumococci.

Pneumococci may, therefore, be distinguished from streptococci by their bile-solubility and, less definitely, their ability to ferment inulin, in addition to their greater pathogenicity for mice and the characteristics of the colonies on agar.

Peroxide is found in considerable quantities of pneumococcus cultures after prolonged incubation because of the lack of catalase in these microorganisms. This, coupled with their sensitivity to peroxide, results in the auto-sterilization of cultures kept in the incubator for many days. Cultures in blood broth, however, remain viable for several weeks in the refrigerator and the bacteria may be preserved for months in the cold in vacuum-desiccated spleens of infected mice.

The pneumococci are more sensitive to the bactericidal activity of the usual antiseptics than are many other bacteria. Soaps, such as ricinoleate and oleate, are pneumococcicidal in relatively high dilutions, 0.04 and 0.004 per cent respectively, and other substances such as phenol and mercuric chloride are also highly effective in the destruction of these bacteria. Quinine and some of its derivatives, such as optochin, are also pneumococcicidal, a fact which has been of interest in connection with the chemotherapy of pneumococcus pneumonia.

Toxins. The severe intoxication observed in pneumococcus infection in man is suggestive of the formation of some toxin by this bacterium. The existence of such a toxin has never been demonstrated, however, and the pneumococcus does not produce a toxin analogous to those of the diphtheria and tetanus bacilli.

Other toxic substances are produced by this microorganism. That there is a hemolysis on blood plates has already been noted, and there is in addition a filterable hemolysin active on sheep, guinea pig and human erythrocytes. The concentrated hemolysin is reported to have lethal and dermotoxic properties.[6] The pneumococcus also produces a leucocidin and a necrotizing substance similar to that formed by some of the staphylococci. Many strains produce hyaluronidase, especially when cultivated in media containing hyaluronic acid.[7] A purpura-producing substance which is non-antigenic and appears to be a cleavage product of pneumococcal protein has been described by a number of workers. Injected into white mice, extracts of pneumococci produce a purpuric condition manifested as a dark blue discoloration of the skin of the feet, tail, ears, nose and genitals.[8]

Although preparations containing these activities have been reported to increase the virulence of relatively avirulent pneumococcus strains when injected simultaneously with the bacteria, the virulence of pneumococci is directly dependent, not on the formation of such toxic substances, but on the production of specific soluble substance and encapsulation.

Classification. The pneumococci are closely related to the streptococci, but the degree of intimacy of the relationship is as yet open to question. Some workers regard these microorganisms as but a species of streptococcus and desig-

[6] Halbert, Cohen and Perkins: Bull. Johns Hopkins Hosp., 1946, 78:340.
[7] See Humphrey: Jour. Path. Bact., 1944, 56:273.
[8] *Cf.* Jultanelle and Reimann: Jour. Exp. Med., 1926, 43:87; *ibid.*, 1927, 45:609.

nate them as *Streptococcus pneumoniae*. In general, however, it is customary to consider the pneumococci as a distinct genus, a practice which is justified by considerations of the sum of the characteristics of the pneumococci and the clinical and epidemiological aspects of the pneumonias. According to Bergey's (1948) classification, the tribe *Streptococceae* is made up of three genera: (1) *Diplococcus*, of which the type species is *Diplococcus pneumoniae* or the pneumococcus; (2) *Streptococcus*, with *Streptococcus pyogenes* as the type species; and (3) *Leuconostoc*, a group of gas-forming, chain-producing cocci found in milk, fermenting vegetables and slimy sugar solutions.

The genus *Diplococcus* includes six species in all, the five in addition to the pneumococcus being obligate anaerobes. *D. paleopneumoniae* closely resembles
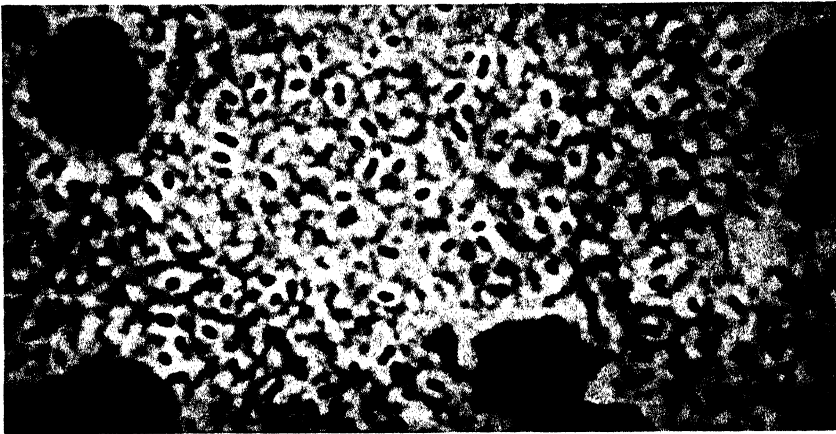
Fig. 53. Pneumococcus in the peritoneal fluid of a mouse. Note the capsules. Fuchsin; × 2200.

the pneumococcus except for its anaerobic character, occurs normally in the buccal-pharyngeal cavity, and is reported to be highly pathogenic. *D. pla-garum-belli* has been found in septic wounds, and the remainder, *D. magnus*, *D. constellatus* and *D. morbillorum*, appear to be normal inhabitants of the mouth and intestinal tract, and have been found in lymphoid tissue such as in the tonsils and appendix.

Pneumococcus Types. A point that has been of somewhat more interest than the formal taxonomic position of the pneumococci is the subdivision of these bacteria into types. The pneumococci contain two types of antigens. One, the so-called somatic antigen, is a constituent of the cell substance proper and is immunologically identical in all pneumococci. The other, the polysaccharide haptene or specific soluble substance (SSS), is type-specific and serves to differentiate the immunological types of pneumococci from one another. The polysaccharide of Type 3 has been the most thoroughly studied chemically (p. 282) and Types 1 and 2 to a lesser extent. These substances have been isolated from most of the other types also. The presence of SSS masks the somatic antigen, and antisera to encapsulated pneumococci are sharply type-specific. These immunological types are sometimes designated by Roman nu-

merals, and the numbering of the types, although purely arbitrary, is a universally accepted convention.

Observations by Dochez and Gillespie[9] showed that the pneumococci found in cases of lobar pneumonia can be divided into four distinct groups, designated as Types 1, 2 and 3 and Group IV, on the basis of specific agglutination and protection tests. Of these, Types 1, 2 and 3, found in the majority of cases of pneumonia (Fig. 54) and producing the most severe types of infection, have specific immunological character, while Group IV is immunologically heterogeneous and is made up of all pneumococci not belonging to the first three types. Through the work of Cooper and her associates of the New York City Department of Health[10] 29 additional serologic types were sorted out of Group IV to make 32 types in all. Of these 26 is closely related to 6 and is sometimes designated 6a, and 15 and 30 are identical or practically so. Six new types and 14 subtypes, related to but not identical with the Cooper types, were described by Kauffmann, Mørch and Schmith[11] who pointed out that the subtypes, while related, were stable and independent. This differentiation was made on the basis of reciprocal absorptions. Seventeen additional types were described by Walter, Guevin, Beattie, Colter and Bucca[12] of which 12 corresponded to 5 of the types and 7 of the subtypes of Kauffmann *et al.* Mørch[13] then later described additional new types, some of which were subtypes. The practice of designating subtypes, *viz.*, 7a, 7b, 7c and so on, has begun to be generally adopted. Under the auspices of the American Public Health Association and the United States Public Health Service, however, Eddy[14] has given individual type numbers to all pneumococcus types and subtypes so far described without regard to the relationships, sometimes very close, between many of the types, making a total of 75 types. Thus the purely arbitrary system of numbering the types without regard to their biological relationships is reverted to and extended.

Although these immunological types are readily distinguished from one another, cross-reactions occur in a number of instances. There appear to be some relations between Types 2 and 5, 3 and 8, 7 and 18, and 15 and 30 which are quite likely due to structural similarities of the polysaccharide haptenes (p. 282). The resemblances are not sufficiently close, however, to invalidate the differentiation of these types. An interesting observation has been that of Forster and Shaughnessy[15] of the occurrence of mixed types, *i.e.*, strains of pneumococci which react with two or more unrelated type specific antisera. Similar observations have been reported by Chinn and Eddy.[16] Such mixed types appear to occur with some frequency as indicated in Fig. 54.

Diplococcus Mucosus. These immunological types are culturally indis-

[9] Dochez and Gillespie: Jour. Amer. Med. Assn., 1913, 61:727.

[10] Cooper, Edwards and Rosenstein: Jour. Exp. Med., 1929, 49:461; Cooper, Rosenstein, Walter and Peizer: Jour. Exp. Med., 1932, 55:531.

[11] Kauffman, Mørch and Schmith: Jour. Immunol., 1940, 39:397.

[12] Walter, Guevin, Beattie, Colter and Bucca: Jour. Immunol., 1941, 41:279.

[13] Mørch: Jour. Immunol., 1942, 43:177.

[14] Eddy: Pub. Health Repts., 1944, 59:449, 451, 485.

[15] Forster and Shaughnessy: Proc. Soc. Exp. Biol. Med., 1940, 44:306.

[16] Chinn and Eddy: Pub. Health Repts., 1941, 56:62.

tinguishable with the exception of Type 3, which stands somewhat apart from the other pneumococci in that it produces a heavy mucoid growth due to its luxuriant capsule formation. It is not infrequently classified as a separate species, *Diplococcus mucosus*. Many cultures show a marked tendency to form chains, and the dividing line between *Diplococcus mucosus* and *"Streptococcus mucosus"* is not a sharp one, if indeed any distinction should be made. These coccoid, heavily capsulated bacteria for the most part ferment inulin and are soluble in bile, so that the tendency is to group them with the pneumococci rather than the streptococci. A few mucoid strains have been reported which are bile insoluble and non-inulin-fermenting.

Pneumococcus Typing.[17] The determination of pneumococcus types is a matter of great practical importance to the therapeutic use of antiserum. The various methods that have been developed are basically immunological but differ in technical detail. The immunological procedures are of three kinds: (1) the agglutination of the pneumococci with type-specific antiserum; (2) the precipitation of SSS with type-specific antiserum; and (3) the *Quellung* reaction. The first two have been discussed in previous sections and need not be considered further here. The *Quellung* phenomenon was described by Neufeld in 1902 and since then has come into general use. A suspension of pneumococci is mixed with undiluted antiserum (rabbit serum is preferable to horse serum) on a slide or cover glass, a small amount of Löffler's alkaline methylene blue is added to facilitate observation, and the preparation is examined under the microscope. In the presence of homologous immune serum there is a marked apparent swelling of the capsule without any obvious change in the size of the bacterial cell itself; no such swelling is observed with heterologous sera. The reaction takes place rapidly and the swelling is usually apparent within a few minutes. The nature of the process of swelling is not known; it is of some interest that it may be reversed by the addition of homologous SSS.[18] The use of serum pools considerably facilitates typing, especially the identification of the higher types. The incidence of the various types determines the most advantageous combinations of antisera. A group of combinations often recommended for use in this country is: (*a*) 1, 2, 7; (*b*) 3, 4, 5, 6, 8; (*c*) 9, 12, 14, 15, 17; (*d*) 10, 11, 13, 20, 22, 24; (*e*) 16, 18, 19, 21, 28; (*f*) 23, 25, 27, 29, 31, 32. Monospecific antisera are, of course, required also. The pneumococcus to be typed is tested with each pool and then with the component antisera of the pool with which it reacts. Thus a pneumococcus may be identified in twelve or less tests.[19]

The sputum may be used directly in the *Quellung* reaction if there are sufficient pneumococci present, or it may be heated and centrifuged and the supernatant used as antigen in a precipitin-ring test (Krumwiede's method). In the latter instance the reaction is dependent upon the presence of SSS in the sputum, and a negative reaction has no significance because of the possible lack of sufficient SSS.

The intraperitoneal inoculation of white mice with sputum results in a

[17] Methods of typing are discussed in detail on pp. 620–640 of Heffron: *Pneumonia*. The Commonwealth Fund, New York. 1939.

[18] Kempf and Nungester: Jour. Inf. Dis., 1942, 71:50.

[19] For pools used in typing all 75 types see Eddy: Pub. Health Repts., 1944, 59:1041.

rapid growth of pneumococci, and peritoneal exudate aspirated after three to six hours contains large numbers of bacteria as well as considerable amounts of SSS. The bacteria may be typed by the *Quellung* reaction, by microscopic slide agglutination (Sabin's method), or by macroscopic agglutination or precipitin tests. When mice are not available the sputum may be cultured in glucose blood broth (Avery's method) and the broth and contained pneumococci used as antigen in the immunological tests.

Variation. The smooth and rough variants that have been found in a variety of bacteria may also be observed in the pneumococcus. As in other cases, there are various intermediate colonial types between the two extremes, and the pneumococcus is virulent in the smooth form and almost completely avirulent in the rough form. The change from smooth to rough is reflected in the microscopic morphology of the cells as a loss of capsule and the ability to form SSS. It was first observed by Stryker[20] that pneumococci cultivated in homologous immune serum did not form capsules; the ability of specific antibody to inhibit the elaboration of capsular substance and thereby render them susceptible to phagocytosis is no doubt an important consequence of the immune response. Since type specificity is determined by the SSS, it follows that the change from smooth to rough is accompanied by a complete loss of type specificity; the somatic antigen is predominant and, irrespective of original type, the pneumococci become immunologically identical. The dissociative change may be reversed, although with difficulty, by animal passage or by cultivating the R form in the presence of anti-R immune serum, or in the presence of heat killed cells from a smooth culture.

Transformation of Types. Of fundamental biological significance, however, was the discovery of Griffith[21] that the inoculation of mice with living R culture mixed with a heat-killed suspension of smooth pneumococci of a type other than that from which the R culture was derived resulted in the conversion of the R variant to the S variety of the new type. These transformations of pneumococcus type have also been brought about *in vitro* by cultivation of R variants in blood broth in the presence of anti-R immune serum and heat-killed smooth pneumococci of heterologous type. The S–R variation occurs in nature (its practical importance to human pneumococcus infections is not clear) and R variants may be isolated from pneumococcus infections in man, but whether the transformation of types likewise occurs in nature is not known. The transforming substance has been shown to be a polymerized ribonucleic acid which is active in inducing type transformation in very small amounts[22] (see p. 182).

Pathogenicity for Man. As indicated above, lobar pneumonia is the most important pneumococcal infection in man. The bacteria are not confined to the lung, however, for they may migrate from this seat of infection through the nasal passages or be distributed via the vascular system to various parts of the body, to give rise to localized foci of infection. Pneumococcemia is of frequent occurrence; available data indicate that 50 per cent is a fair estimate. A number of workers emphasize the prognostic value of blood culture, and in

[20] Stryker: Jour. Exp. Med., 1916, 24:49.
[21] Griffith: Jour. Hyg., 1928, 27:113.
[22] McCarty: Bact. Rev., 1946, 10:63.

most instances the case fatality is considerably higher when pneumococci are present in the blood stream. Avery, Chickering, Cole and Dochez[23] observed a case fatality of 55.8 per cent in those with positive blood cultures and 8.3 per cent of patients with negative culture. Similar results have been reported by others.

Among the pathologic processes that occur as complications and sequelae of pneumococcus pneumonia, or, it may be noted, as independent and primary affections, are inflammations of the pleura, pericardium and meninges. Meningitis and otitis media are frequently secondary to pneumonia, and the connec-
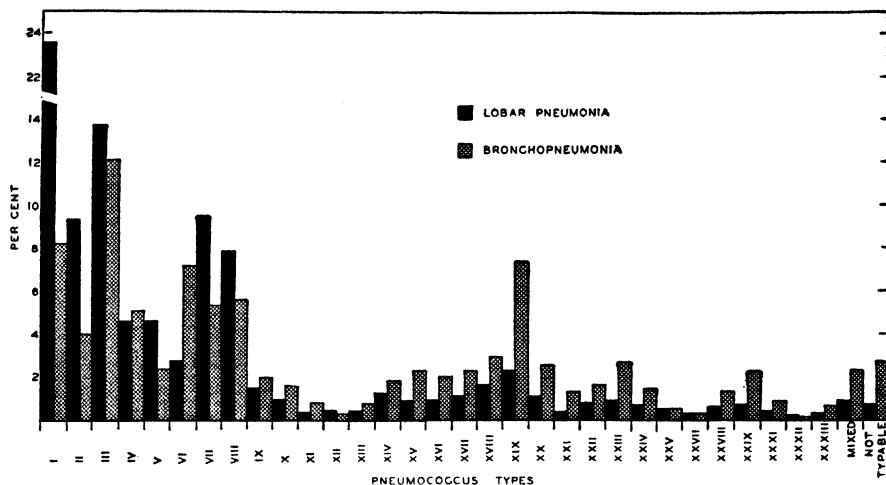
Fig. 54. The incidence of pneumococcus types in lobar and bronchopneumonia observed in a two-year survey in California, Colorado, Illinois, Louisiana, New Jersey and Missouri in which pneumococci from 12,447 cases of lobar pneumonia and 3847 of bronchopneumonia were typed. The relative predominance of Type III over Type II is sometimes observed. Note the more common occurrence of higher types in bronchopneumonia, the relative incidence of mixed types, *i.e.*, those reacting to more than one antiserum, and the incidence of types other than the first thirty-three. Data from Rumreich *et al.*: Pub Health Repts., 1943, 58:121.

tion between inflammation of the middle ear and meningeal infection has often been noted. To those inflammatory conditions of pneumonococcal origin might be added a long list of others provoked by the same organism. There appear to be few, if any, organs or tissues that are not under some circumstances subject to attack. Sinusitis, parotitis, conjunctivitis, enteritis and a great variety of other affections are occasionally due to the activity of pneumococci. In general, pneumococcus infections of this type tend to a more favorable outcome than similar infections with streptococci or staphylococci.

Resistance to pneumococcal infection is, in large part, a matter of individual predisposition, and the mere presence of pneumococci in the upper respiratory tract is not alone sufficient to bring about pulmonary infection. Specific immunity plays a negligible part in resistance, but the complex of factors as-

[23] Avery, Chickering, Cole and Dochez: *Acute Lobar Pneumonia.* Monographs of the Rockefeller Institute for Medical Research No. 7, 1917.

sociated with a state of physiological well-being is of the greatest significance. A preliminary depression of resistance by other infections, severe or sudden exposure to cold, fatigue and other predisposing factors, is an almost invariable preliminary to pneumococcus infection. The part played by pneumococcus pneumonia in the fatal termination of many diseases, for example, is well known.

The Pathogenicity of Pneumococcus Types. The case fatality of pneumococcus pneumonia is relatively high and indicative of the pathogenicity of these bacteria once they have become established in the lungs. The pneumococcus types differ from one another in this respect; the case fatality in Type 1 infections is 25 to 30 per cent, that of Type 2 about 40 per cent, that of Type 3, 40 to 60 per cent, and that of Group IV infections perhaps 15 to 20 per cent. Because of the relatively recent differentiation of the types comprising Group IV, data on case-fatality rates for these types are very meager as yet. The frequency of occurrence of the pneumococcal types in lobar pneumonia and bronchopneumonia is given in Fig. 54. Here the ten leading types are: 1, 2, 3, 4, 5, 6, 7, 8, 14 and 19. In some series Type 2 is more frequent than Type 3 and in others, including this one, the reverse is true.

Pneumococcus Carriers. As a strict parasite, the pneumococcus is found in man rather than in his environment. Healthy carriers of these bacteria are common; 40 to 60 per cent of groups of persons examined have been found to harbor pneumococci in the upper respiratory tract. This proportion is variable, however, being relatively high during the cold months of the year and higher among groups of contacts than among non-contacts. The carrier state is not permanent but rather sporadic and intermittent; many persons may carry these bacteria for a short time, particularly while having colds and other infections of the upper respiratory tract, while others may carry them for longer periods. There is also a seasonal fluctuation in that increased numbers are found in the winter months.

The great majority of pneumococci found in carriers are the relatively less virulent types of Group IV. Types 1, 2 and 3 are much less frequently found, but an appreciable portion of the population may harbor these types at any one time (see table). In the study made by Smillie, Calderone and Onslow[24] almost every type of pneumococcus was encountered; many individuals carried two or more types simultaneously. The types most commonly found were 3, 7, 21, 25 and 11 in that order of frequency. It is not clear whether an appreciably effective immunity is developed by carriers.[25]

The Epidemiology of Pneumococcus Pneumonia.[26] Pneumococci are disseminated chiefly through the secretions and discharges of the mouth and upper respiratory tract by direct contact between persons. Droplet infection (p. 231) undoubtedly plays a large part in the transmission of these microorganisms and perhaps accounts for the seasonal incidence of the disease (Fig. 55) and the increased frequency of carriers during the cold months of the year.

While it is obvious that pneumococcus infection is always exogenous in the

[24] Smillie, Calderone and Onslow: Amer. Jour. Hyg., 1943, 37:156.
[25] Cf. Finland, Brown and Barnes: Amer. Jour. Hyg., Sec. B, 1940, 32:24.
[26] See the review by Finland: Medicine, 1942, 21:307.

last analysis, for practical purposes it is probably endogenous in a large proportion of cases. With a high carrier rate pneumococci are frequently present in the normal individual, and when resistance is reduced to a sufficiently low level they are able to set up an infection. The high incidence of pneumococci of Types 1 and 2 (Fig. 54) as contrasted with the low carrier incidence of these types, together with the occurrence of seeming epidemics of pneumococcus pneumonia on a small scale, has been regarded by some as evidence of exogenous infection. It is more probable, however, that the greater virulence of some pneumococcus types operates as a selective factor to disturb the random distribution of types in pneumococcus pneumonia. It is known, for example, that minor respiratory infections may be common or epidemic in small groups

PNEUMOCOCCUS CARRIERS*

Persons Examined			Incidence of Carriers				
	Total	Cases Found		Total†	Per Cent Incidence of Types		
		Number	Per Cent		1	2	3	Group IV
Non-contacts....	2332	1000	42.9	1027	0.5	0.9	8.4	34.2
Contacts........	1782	977	54.8	1018	3.3	2.7	10.0	41.0

* Modified from Heffron's[17] data.
† The incidence of types is greater than the incidence of carriers because in some instances more than one type was found.

such as a family. If a given type of pneumococcus of high virulence invades and spreads within the group so that a high proportion of the individuals become carriers, the operation of factors which reduce resistance in the group may result in one or more members coming down with pneumonia due to the type carried. Smillie and Jewett[27] observed just such a sequence of events in a group of children in a nursery. The group was invaded by a virulent Type 14 pneumococcus which caused no harm but when the individual developed an acute respiratory infection, the dormant pneumococcus spread to the middle ear, conjunctivae and lungs. Such children were sent to the hospital ward and carried the pneumococcus which spread to most of the children there. Again, the infection was activated, sometimes with very serious consequences, on the development of respiratory infection.[28]

Other epidemiological characteristics of pneumococcus pneumonia include seasonal incidence (Fig. 55), which corresponds roughly with the carrier rate; the age incidence, characterized by high morbidity and mortality in infants and the aged; the higher incidence in males than in females; and the apparent greater susceptibility of the Negro as contrasted with the white race.

[27] Smillie and Jewett: Amer. Jour. Pub. Health., 1942, 32:987.
[28] For a detailed experimental study of epidemic pneumonia see Hodges and MacLeod: Amer. Jour. Hyg., 1946, 44:183, 193, 207, 231, 237.

Bacteriological Diagnosis of Pneumococcus Infections. The pneumococcus may be isolated by culture or animal inoculation from specimens such as sputum, pleural exudate, blood, spinal fluid, pus, etc. Blood agar is the medium of choice, the bacteria growing up in twenty-four hours as small colonies surrounded by a zone of green hemolysis. It is not possible to distinguish them from *a* hemolytic streptococci by colonial or microscopic morphology but differentiation may be made by the fermentation of inulin and bile solubility of the pneumococcus and its immunological reactions. Blood specimens are cultured in buffered dextose veal infusion broth, containing 5 mg. per 100 ml. of *p*-aminobenzoic acid if the individual is undergoing sulfa drug therapy, as in the case of culture of the streptococci. A portion of
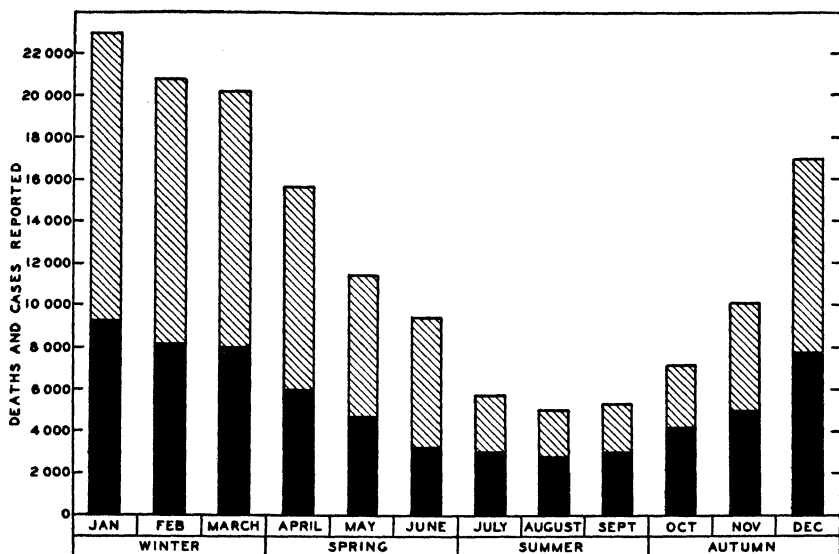
Fig. 55. The seasonal incidence of pneumococcus pneumonia. Averages of numbers of reported cases and deaths by months for the years 1939 to 1945 inclusive. Data from Supplements to Public Health Reports.

sputum, washed in three changes of sterile saline and emulsified in saline, may be inoculated intraperitoneally in a white mouse. With virulent strains the animal will show signs of illness in five to eight hours and microscopic examination of smears of peritoneal exudate will show large numbers of encapsulated diplococci.

The pneumococcus is identified and typed with antiserum, usually by the *Quellung* reaction through agglutination; precipitin tests may be used also. When large numbers of the bacteria are present in sputum, the typing may be done directly without culture or mouse inoculation, but since the *Quellung* reaction is inhibited in the presence of large amounts of SSS, negative reactions are not significant. Typing is readily carried out, as indicated earlier, on pneumococci present in mouse peritoneal exudate or cultures.

Pathogenicity for Lower Animals. The susceptibility of the usual laboratory animals to pneumococcus infection is variable, ranging from the highly

susceptible mouse and rabbit through the less sensitive guinea pig to the cat, dog, chicken and pigeon, which are highly resistant. Rare instances of naturally occurring infections in the usual experimental animals have been reported; such a spontaneous epizootic of type 19 pneumococcus infection in the guinea pig was reported by Homburger *et al.*[29] Animal experiments with the pneumococcus present an example of the general law that susceptibility is characterized by general septicemic infection, resistance by the occurrence of a localized process. The mouse and the rabbit develop a rapidly fatal septicemia, and in these animals lung lesions, when they occur at all, are slight and usually limited to the bronchopneumonic type. It is possible to produce typical lobar pneumonia in the rabbit by carefully balancing the susceptibility of the animal and the virulence of the bacterium through the use of attenuated cultures or previous partial immunization. Resistant animals, such as the dog, show an approximation toward the type of pneumococcus infection observed in man, and lobar pneumonia may be produced in monkeys by intratracheal inoculation.[30] The lesions produced in monkeys were considered identical with those in human lobar pneumonia. It is of some interest that pneumococci were found in the blood within six hours after their introduction into the trachea and before the signs of pneumonia appeared, suggesting the bronchogenic rather than the hematogenous origin of the infection.

Man, therefore, may be regarded as an animal of rather high normal resistance. This resistance may, however, be so reduced as to permit the production of localized manifestations, which in still more susceptible individuals may lead to a fatal septicemia. In some cases death is due to overwhelming interference with respiration caused by the local pulmonary lesions; in others, to a general systemic poisoning or toxemia.

Immunity. Experimental animals may be actively immunized against pneumococcus infection by the injection of vaccines of the smooth, virulent bacteria, although the immunity is not of long duration, *i.e.,* not more than a few months. The development of the immune state is accompanied by the appearance of antibodies, precipitins, agglutinins and the like, as well as a protective quality in the blood serum. In man the situation is somewhat obscure; there is undoubtedly an intimate relation between recovery and the appearance of humoral antibodies, but immunity following infection is slight and transitory, and one attack may succeed another after a short interval. Active immunity in experimental animals is type-specific, however, and it is not improbable that under natural conditions different immunological types may participate in successive attacks.

The question of active immunization of man to pneumococcus infection is of continued interest. Results suggestive of its value were obtained from mass inoculation studies[31] in 1918–1919 in this country, and from the extensive studies of Felton and his co-workers.[32] Heidelberger *et al.*[33] have shown that the inoculation of type-specific polysaccharides induces an antibody

[29] Homburger, Wilcox, Barnes and Finland: Science, 1945, *102*:449.
[30] Blake and Cecil: Jour. Exp. Med., 1920, 31:403.
[31] Cecil: Medicine, 1925, 4:395.
[32] *Cf.* Felton *et al.*: Pub. Health Repts., 1941, 56:1041 *et ante.*
[33] Heidelberger, MacLeod, Kaiser and Robinson: Jour. Exp. Med., 1946, 83:303.

response which reaches a peak within six weeks and persists for about six months; booster inoculation during the following eighteen months was not effective in raising the diminishing titers. MacLeod *et al.*[34] found that such immunization significantly reduced both carrier rate and cases of infection with the pneumococcus types immunized against, though not the incidence of other types. In the aggregate, then, these data suggest that an appreciable degree of effective immunity may be produced by immunization procedures.

The results of the therapeutic use of antiserum are variable, being excellent with some types of pneumococci and not with others. The treatment of Type 1 infections with specific antiserum is relatively successful in that the case fatality is reduced from 25 to 30 per cent to 12 per cent. Type 2 infections do not respond quite so well to serum therapy, the case fatality being reduced from about 40 per cent to 25 per cent, and Type 3 infections are little benefited. Of the types comprising Group IV, Types 5 and 7 appear, on the basis of present evidence, to respond to serum therapy. Specific serum therapy is complicated by the existence of the multiplicity of higher types of pneumococci. Diagnostic and therapeutic antisera have been made available commercially for thirty-two types of pneumococci, but it seems probable that polyvalent sera, based on the frequency of occurrence of the higher types, will become a practical necessity.[35] Combinations to give such polyvalent antisera have been suggested by Eddy.[36]

Sulfonamide and penicillin therapy has, to a large extent, supplanted serum therapy. Antiserum is generally prepared in horses, but some observations[37] have suggested that rabbit antisera may be more effective, in part because higher protective titers are obtainable than with horse serum and in part because the smaller antibody molecules (p. 309) should facilitate absorption. Pneumococcus antisera are standardized by titration of mouse protective antibody.[38]

It may be noted here that a bacterial enzyme decomposing the Type 3 specific polysaccharide isolated by Avery and Dubos[39] protects experimental animals against Type 3 pneumococcus infection.[40] The injection of the enzyme preparations, however, produces untoward reactions, notably a febrile reaction and leucopenia, and therefore they have not been used in man.

[34] MacLeod, Hodges, Heidelberger and Bernhard: Jour. Exp. Med., 1945, 82:445.

[35] See the comprehensive review of this matter by Finland: Jour. Amer. Med. Assn., 1942, *120*:1294.

[36] Eddy: Pub. Health Repts., 1944, 59:1485.

[37] Horsfall, Goodner and MacLeod: Science, 1936, 84:579; Jour. Amer. Med. Assn., 1937, *108*:1483; Goodner, Horsfall and Dubos: Jour. Immunol., 1937, 33:279.

[38] *Cf.* Military Surgeon, 1944, 94:386.

[39] Avery and Dubos: Science, 1930, 72:151.

[40] Avery and Dubos: Jour. Exp. Med., 1931, 54:73; Goodner, Dubos and Avery: Jour. Exp. Med., 1932, 55:393.

THE GRAM-NEGATIVE PATHOGENIC COCCI (NEISSERIA): THE GONOCOCCUS AND THE MENINGOCOCCUS

The gonococcus and the meningococcus are the chief representatives of a small group of closely related bacteria whose other members are nonpathogenic inhabitants of the mouth and upper respiratory tract of man. Two groups of species are separated from one another on the basis of pigment production, and further differentiation is made by means of fermentation reactions.

FERMENTATION REACTIONS OF THE GRAM-NEGATIVE DIPLOCOCCI

Non-pigmented Species	Dextrose	Maltose	Sucrose	Levulose	Mannitol
N. gonorrheae (gonococcus)	+	−	−	−	−
N. intracellularis (meningococcus)	+	+	−	−	−
N. catarrhalis	−	−	−	−	−
N. sicca	+	+	+	+	−
Pigmented Species					
N. perflava (flava I)	+	+	+	+	+
N. flava (flava II)	+	+	−	+	−
N. subflava (flava III)	+	+	−	−	−
N. flavescens	−	−	−	−	−

Pigmented varieties are frequently found in the nasopharynx (*Neisseria flava* I, II, III).

THE GONOCOCCUS [1]

Neisser[2] in 1879 first called attention to the constant presence of a peculiar coccus in gonorrheal pus. In cases of gonorrhea of recent origin this was the sole organism found; it not only occurred in the urethral and vaginal discharges of ordinary gonorrhea, but was present in the exudate in conjunctivitis

---

[1] Present knowledge of gonorrhea is reviewed in the report of the Committee for Survey of Research on the Gonococcus and Gonococcal Infection. Thomas and Bayne-Jones: Amer. Jour. Syph., 1936, 20: Suppl. to No. 1.

[2] Neisser: Centralbl. f. d. med. Wissensch., 1879, 17:497.

due to gonorrheal infection. Pure cultures of this microorganism were isolated in 1885 by Bumm,[3] who succeeded in demonstrating its etiologic relation to gonorrhea by the inoculation of human volunteers. This bacterium, known generally as the *gonococcus,* has been termed *Micrococcus gonorrheae* and *Diplococcus gonorrheae,* but the genus *Neisseria* is now more or less generally accepted and this bacterium is properly known as *Neisseria gonorrheae.*

Morphology and Staining. In preparations made from gonorrheal pus the cells of the gonococcus occur in pairs, with the flattened sides in juxtaposition; the appearance in stained preparations resembles that of a coffee bean. In pure culture the cocci appear as oval or spherical and are often aggregated in irregular masses without the typical diplococcus arrangement. In pus smears the gonococcus occurs almost entirely within the leucocytes; frequently enormous numbers may be found packed within a single phagocyte. In the earliest stages of infection, however, gonococci may be found extracellularly, and the
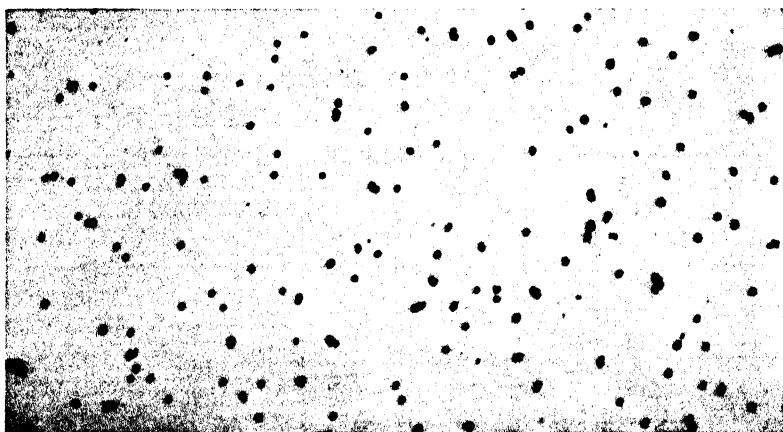
Fig. 56. The gonococcus from pure culture. Fuchsin; × 1050.

same is true of cases of gonorrhea of long standing. The gonococcus is non-motile and does not form spores.

The colonies of the gonococcus are small, translucent, finely granular with lobate margin and grayish white with a pearly opalescence when viewed by transmitted light. Larger colonies may be formed on special media. Colonial appearance is, however, subject to considerable variation (see below).

Unlike the pyogenic cocci, the gonococcus and related forms are gram-negative, a staining characteristic that is of considerable diagnostic value since it serves to differentiate the gonococcus from other cocci present in the urethral or vulvovaginal tracts. Other gram-negative cocci may be found occasionally, sometimes within the leucocytes, but they are rare. The tendency to decolorization in the gram stain is variable. Some strains decolorize much more readily than others, and gonococci embedded in masses of pus may retain the stain; hence the preparation of thin, uniform films is highly desirable. The

[3] Bumm: *Der Mikroorganismus der gonorrhoischen Schleimhauterkrankungen.* Wiesbaden. 1885.

gonococcus stains with the aniline dyes, but polychrome stains, such as Pappen-heim's stain,[4] are more useful. Intracellular granules may be found in stained preparations, but in general the gonococci from young cultures stain evenly, while older cultures (twenty-four hours and older) contain large swollen in-volution forms which may stain poorly.

Physiology. In its nutritive requirements the gonococcus is one of the most fastidious bacteria, particularly upon primary isolation. An enriched me-dium is, of course, required for cultivation. Earlier media were enriched by the addition of ascitic and hydrocele fluid. A proteose–3 hemoglobin agar has been used to some extent in this country, but the most satisfactory medium is chocolate (heated blood) agar prepared with an infusion base. Nutritional requirements appear to be highly complex and are not well known. Mueller and Hinton[5] have devised a protein-free hydrolyzed casein-starch medium that may be autoclaved and Boor[6] has been able to grow the gonococcus on a medium containing tryptic digest of casein, cystine, dextrose and buffer. The cystine appears to be required by the gonococcus but not by the meningo-coccus. Growth is markedly stimulated by glutathione in many cases, and in others strains appear to require it. Synthetic media have been devised by Wel-ton, Stokinger and Carpenter[7] and by Gould, Kane and Mueller[8] which contain amino acids, magnesium, iron, etc., and will support growth of the gono-coccus. A sufficient supply of moisture is essential, there should be water of condensation on the tubes or plates and the atmosphere of the incubator should be kept saturated with water. Incubation in an atmosphere of increased CO_2 tension, about 10 per cent, greatly improves growth and is a practical necessity in primary isolation. With continued cultivation on laboratory media, the gonococcus appears to become somewhat less fastidious and some strains may eventually grow upon the ordinary infusion media. The preservation of cultures is difficult, however, for the gonococci die off in two to three days at room temperature and in six to eight days at 37° C. but will live longer when kept in the cold. Even upon continued transfer the gonococci die off and cul-tures are frequently lost. The optimum temperature for growth is 37° C., growth does not occur below 30° C., and temperatures of 40° to 41° C. are definitely harmful. The gonococcus will grow sparsely under anaerobic con-ditions but is essentially aerobic in character.

With respect to deleterious influences the gonococcus is a delicate micro-organism. It is readily killed by heat as indicated above and by dilute anti-septics; 1 per cent phenol, for example, kills in one to three minutes. It is remarkably sensitive to certain of the flavine dyes (p. 149) and is rapidly destroyed by silver salts. The gonococcus is sensitive to drying and, under ordi-nary conditions, can survive exposure to the air for only a very short time—one to two hours—although in masses of dried pus it may live exceptionally for six to seven weeks.

The gonococcus is not very active biochemically. Glucose is fermented, prin-

[4] A methyl green-pyronine stain. For recent studies on this stain see Barritt: Brit. Med. Jour., 1944, i:494.
[5] Mueller and Hinton: Proc. Soc. Exp. Biol. Med., 1941, 48:330.
[6] Boor: Proc. Soc. Exp. Biol. Med., 1942, 50:22.
[7] Welton, Stokinger and Carpenter: Science, 1944, 99:372.
[8] Gould, Kane and Mueller: Jour. Bact., 1944, 47:287.

cipally to lactic acid, but many other sugars are not attacked, indol is not produced, nitrates are not reduced, and no change is produced in litmus milk. Catalase is produced, and a characteristic that has been turned to practical differential use is the formation of indophenol oxidase. McLeod *et al.* recommend the cultivation of suspected material on 10 per cent heated blood (chocolate) agar in an atmosphere containing 8 per cent carbon dioxide, followed by twenty-four hours' incubation in air. A 1 per cent solution of tetramethyl-*p*-phenylenediamine is poured on the incubated plate and poured off again immediately or sprayed on with a nasal atomizer. Colonies of bacteria forming indophenol oxidase turn a bright purple color. The bacteria are not immediately killed and may be subcultured within half an hour. This so-called

Fig. 57. Colonies of the gonococcus on blood agar. × 6.

"oxidase reaction," coupled with the examination of smears for gram-negative intracellular diplococci, is becoming generally used in the laboratory diagnosis of gonorrhea.

Toxins. Although a number of attempts were made by the early workers to demonstrate the formation of toxins by the gonococcus, variable results were obtained. These bacteria form a weak hemolysin, and their cell substance will kill laboratory animals when injected in sufficient amount and will evoke suppuration upon instillation into the human urethra. A carbohydrate-lipid complex has been prepared by Boor and Miller[9] by extraction of the cells with M/2 trichloracetic acid which is both antigenic and toxic to mice and may be regarded as an endotoxin.

Variation. Numerous workers have observed that the cultural characteristics of the gonococcus are subject to considerable variation. It has been found[10] that two types of colonies may be observed which appear to be correlated with immunologic type. The one, designated as Type I, is a large, ir-

[9] Boor and Miller: Jour. Inf. Dis., 1944, 75:47.
[10] Atkin: Brit. Jour. Exp. Path., 1925, 6:235.

regular, flattened, translucent colony that, on continued incubation, gives rise to surface papillae; the other type of colony, Type II, is somewhat smaller, round, raised with slightly convex uneven surface, and opaque. Type I colonies are generally recovered from acute gonorrhea, while Type II are found in old laboratory strains and, sometimes, in chronic gonorrhea. The papillae are thought to represent the first step in transition toward the Type II form. The relation of these forms to the dissociative changes of other bacteria is uncertain. Small colony variants, presumably arising from a dissociative process, have been produced experimentally[11] and have also been found on primary isolation from clinical material.[12]

Classification. The relationship of the gonococcus to the other members of its genus has already been discussed and need not be considered further. The gonococci are immunologically heterogeneous, and a number of attempts have been made to subdivide them into types. Most of the freshly isolated strains from acute cases appear to fall in one serological group, while old stock cultures and strains from chronic cases constitute a second subdivision which is regarded by some as a degenerate form. "Intermediate" and serologically "independent" strains occur, and a sharp differentiation may not be made. The chemical fractionation of gonococci has indicated the presence of polysaccharide and nucleoprotein components which are shared by other species of Neisseria, the meningococcus and *Neisseria catarrhalis*.[18] Type-specific carbohydrate has been found in gonococci which is said to be responsible for the immunological character of Type I and II, the two groups noted above. Specific agglutinating antisera may be prepared in rabbits and chickens by the intravenous inoculation of living gonococci. The significance of these findings to gonococcal infection is as yet uncertain.

Pathogenicity for Man. Few diseases are so widely disseminated through all classes of society as gonorrhea. Precise information as to the incidence of the disease is not available; a morbidity rate of 10 per cent in the United States is generally accepted as a conservative estimate. It is calculated that about a million fresh gonococcal infections occur each year, and there is no evidence of a downward trend.[14]

As a rule the gonococcus attacks primarily the human urethra and gives rise to an inflammation which may be followed by chronic urethritis and stricture. There is a marked tendency for spread of the infection along contiguous mucous surfaces, resulting, in the male, in epididymitis and other inflammatory conditions. In the female, the entire genito-urinary tract may be involved, and the fallopian tubes, the ovaries and the peritoneum are not uncommonly invaded. The gonococcus may also invade the blood stream from local lesions and be carried to various parts of the body and give rise to a variety of extra-genital lesions. Especial predilection is shown for the synovial membranes of the joints, where it causes the so-called "gonorrheal rheumatism," and for the heart valves, where it produces endocarditis. Local or general complications

[11] Raven: Jour. Inf. Dis., 1934, 55:328.
[12] Morton and Shoemaker: Jour. Bact., 1945, 50:585.
[18] Boor and Miller: Jour. Exp. Med., 1934, 59:63; Miller and Boor: Jour. Exp. Med., 1934, 59:75.
[14] Vonderlehr and Usilton: Amer. Jour. Syph., 1938, 22:537.

occur in perhaps 30 per cent of all cases. Gonococcal meningitis occurs, perhaps more frequently than formerly thought.[15]

Once established, gonococcal infection persists for a long time; five to fifteen years' duration has been reported, but exclusion of reinfection is a difficult matter. Carpenter and Westphal,[16] however, have observed infection of seven years' duration without reinfection. Following symptomatic cure by chemotherapy, gonococci may persist in the urethral secretions; Koch, Mathis and Geiger[17] found, for instance, that nearly one-third of a group of 926 patients followed after apparent cure continued to carry gonococci. In general, very little is as yet known of the gonococcus carrier or the part played by the carrier in the spread of the disease.

Fig. 58. Urethral smear from gonorrhea. Gram stain. Note the intra- and extra-cellular position of the gonococci and their typical coffee-bean shape and arrangement in pairs. × 2400.

Gonorrheal vulvovaginitis occurs in epidemic form in little girls, and in these instances the infection is transmitted by bedclothes, towels, common bathtubs and other fomites. The commonest complications are urethritis, proctitis and cervicitis. Such epidemics are frequently exceedingly difficult to control and constitute a serious problem in many institutions such as children's wards in hospitals.[18]

Gonorrheal ophthalmia of the newborn is a well-known consequence of maternal infection; infection does not occur *in utero* but during passage through the birth canal. Although exact information is not obtainable, it is estimated that 10 per cent of all cases of blindness are traceable to this source, and that in the United States there are perhaps 12,000 children blind from this cause. The instillation of silver nitrate or other silver salts immediately after birth prevents infection.

[15] Branham, Mitchell and Brainin: Jour. Amer. Med. Assn., 1938, *110*:1804.

[16] Carpenter and Westphal: Amer. Jour. Pub. Health, 1940, *30*:537.

[17] Koch, Mathis and Geiger: Ven. Dis. Inf., 1944, *25*:35.

[18] A comprehensive discussion of vulvovaginitis in children may be found in the Medical Officer, 1938, *59*:191, 203; see also Cohn, Steer and Adler: Ven. Dis. Inform., 1940, *21*:208.

Except in the case of vulvovaginitis in children, gonorrhea is spread by direct contact, usually sexual. Once infected, an individual may remain infective for a long time, and gonococci may persist in the genito-urinary secretions for years after apparently complete recovery, and even though they may not be found by bacteriological examination the infection may be transmitted. Gonorrhea persists, then, in the human population in a smoldering endemic form and, as a consequence of the nature of its transmission, widespread epidemics in the usual sense do not occur.

Bacteriological Diagnosis of Gonococcus Infection.[19] The demonstration and identification of the gonococcus are essential to the diagnosis of gonorrhea and it should be borne in mind that every case of gonorrhea is potentially a medico-legal case. The presence of gram-negative intracellular diplo-
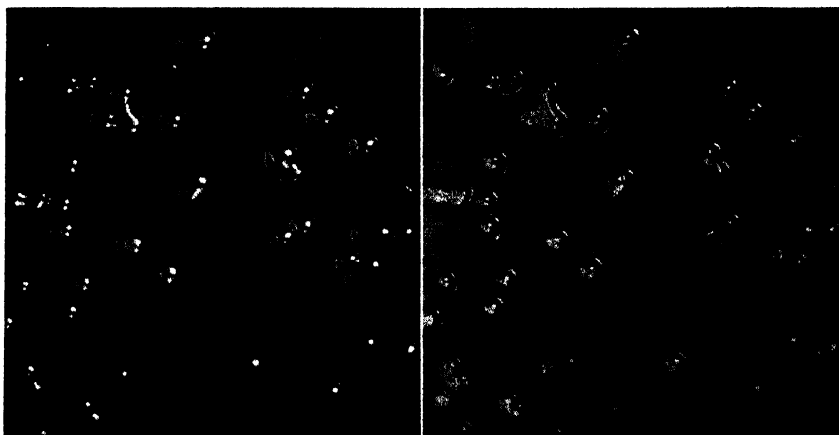
Fig. 59. The oxidase test for the identification of gonococcus colonies. Pure culture on blood agar. Left, gonococcus colonies before the application of tetramethyl-*p*-phenylenediamine solution. Right, the same colonies after the application of the reagent. Note the greater intensity of color about the edges of the colonies immediately after application, and the discoloration of the medium. × 5.

cocci in urethral smears in most cases justifies a provisional diagnosis of gonorrhea; as a rule gonococci may be found readily in acute infections, but in chronic infections, especially in the female, demonstration of the organisms by direct smear or culture is usually difficult. Morphologically similar bacteria may, however, be present in the vagina or conjunctivae. It is generally agreed that culture of the gonococcus gives a higher proportion of positive results than direct smear examination alone and cultured gonococci may, of course, be identified. The question of survival of this relatively delicate bacterium in transport of specimens is of some importance. No really satisfactory method of preserving specimens has been developed. Of the more recent work, it may be noted that Peizer and Steffen[20] found that immediate streaking of the swab on plasma hemo-

[19] For a recent and detailed summary of the laboratory procedures see Carpenter: Ven. Dis. Inform., 1943, 24:133; also the summary of a panel discussion in Amer. Jour. Pub. Health, 1947, 37:1461.

[20] Peizer and Steffen: Ven. Dis. Inf., 1947, 28:218.

globin agar in a screw cap bottle resulted in a high proportion of positive cultures, and these workers are of the opinion that the inclusion of dyes such as Nile blue or crystal violet in the medium may destroy gonococci as well as contaminants. For culture an infusion chocolate agar, developed by McLeod, or minor modifications of it such as the plasma hemoglobin modification studied by Thayer, Schubert and Bucca,[21] is a medium of choice, and is inoculated directly with the specimen (or sediment if it is urine or spinal fluid). The culture must be incubated in 10 per cent CO_2; this atmosphere may be satisfactorily approximated by putting the plates in a jar together with a lighted candle and sealing or by the inclusion of a handful of moistened fresh oats in a sealed container with the cultures. The oxidase test serves to differentiate the oxidase-positive colonies and, if picked immediately, they may be subcultured. Identification is based upon sugar fermentations in serum broth.

Pathogenicity for Lower Animals. The gonococcus is non-pathogenic for lower animals, aside from the toxicity of the cell substance as noted above, and gonorrhea has never been reproduced in experimental animals, including anthropoid apes. An experimental infection of the anterior chamber of the rabbit's eye has been described by Miller and his co-workers[22] in which the gonococci multiply and invade intraocular tissues, especially the ciliary body and lens, giving rise to a chronic infection in approximately one-third of the animals. This infection has been made use of in the study of the efficacy of chemotherapeutic agents, etc.

Immunity. Little if any immunity to the gonococcus is acquired as a result of infection, and second and third infections may be superimposed upon the first, *i.e.*, acute upon old chronic infections. As might be expected, then, the therapeutic use of vaccines and various types of antisera is without effect. The significance of the observed extensive phagocytosis of gonococci by polymorphonuclear leucocytes is uncertain.

Some degree of immunological response is evident, however. Complement-fixing antibodies are usually present, and patients may give a marked skin reaction to suspensions of killed gonococci. A number of attempts have been made to utilize these responses in the immunological diagnosis of gonorrhea.[23] The complement-fixation test has shown some promise but is not generally used. The skin reaction is apparently too variable to have practical value.[24] It has also been observed that the discharges from gonorrheal inflammation give a precipitin reaction with antigonococcus serum, but this flocculation reaction has as yet no diagnostic value.

<div align="center">

THE MENINGOCOCCUS[25]

</div>

Inflammation of the meninges or investing membranes (pia-arachnoid) of the brain and spinal cord may be provoked by a variety of microorganisms, and may occur either as a primary affection or secondarily in the train of an infection originally begun elsewhere. One form of meningitis, characterized

---

[21] Thayer, Schubert and Bucca: Ven. Dis. Inf., 1947, 28:37.

[22] Miller *et al.*: Jour. Inf. Dis., 1945, 77:193, 201, 216.

[23] *Cf.* Casper: Ven. Dis. Inform., 1941, 22:119.

[24] *Cf.* Torrey: Jour. Immunol., 1940, 38:413.

[25] For general reviews see Murray: Med. Res. Council Spec. Rept., Ser. No. 124, 1929; Branham: Bact. Rev., 1940, 4:59.

especially by epidemic spread and usually designated as *epidemic cerebrospinal meningitis,* spotted fever or cerebrospinal fever, is caused by a specific micro-organism commonly known as the meningococcus.

This bacterium was described by Marchiafava and Celli in the meningeal exudate as early as 1884, but the first important work upon it was that of Weichselbaum, who, in 1887, obtained it in pure culture and described it in detail as the characteristic micrococcus found in six cases of acute cerebro-spinal meningitis. Confirmation was supplied by the work of Jäger in spite of some faulty observation. The etiologic role of the meningococcus has since been securely established by a number of investigations.[26]

The meningococcus has been designated by a variety of names, including *Micrococcus meningitidis, Micrococcus intracellularis meningitidis, Neisseria meningitidis* and, according to Bergey (1948) *Neisseria intracellularis.* Al-
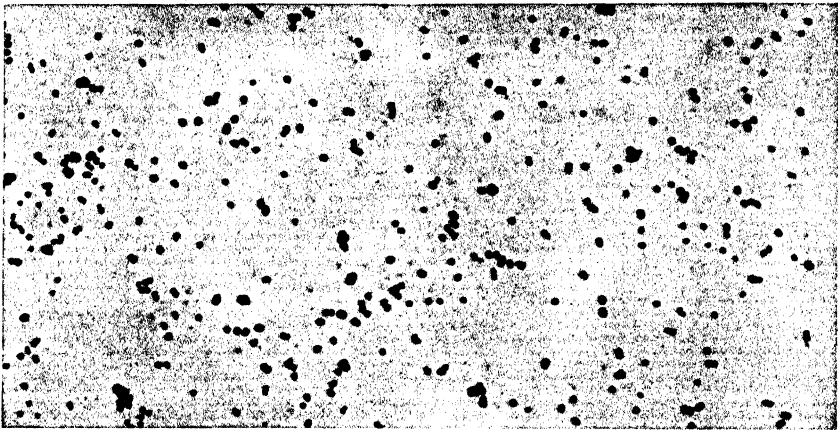
Fig. 60. Meningococcus, pure culture. Note the typical diplococcus arrangement. Fuchsin; × 1050.

though *Neisseria* is generally accepted, common usage is divided between *intracellularis* and *meningitidis.*

Morphology and Staining. In film preparations of the meningeal exu-date the meningococcus is very like the gonococcus and occurs in pairs or tetrads both within the leucocytes and free. The diplococci are flattened toward one another like gonococci, and there is considerable variability in the size of different cells in the same smear. In cultures the meningococcus aver-ages a little less than 1 μ in diameter, and appears, as a rule, in pairs; short chains are seen more rarely. The variability in size observed in meningeal exudate may also be seen in cultures, particularly in those more than twenty-four hours old. Involution forms are common, and it is not unlikely that the larger cells are degenerative. Capsules are usually not apparent but become swollen in the presence of specific immune serum—the *Quellung* reaction. The meningococcus is non-motile and does not form spores.

Meningococcus colonies in blood agar are moist, elevated, smooth and with

[26] *Cf.* Foster and Gaskell: *Cerebrospinal Fever.* Cambridge University Press, London. 1916.

a bluish gray tinge. They do not produce green discoloration or hemolysis
and may be readily differentiated from the hemolytic and viridans strepto-
cocci and the pneumococcus. The colonies are not so white and opaque as
those of the staphylococci.

The meningococcus stains readily with the usual aniline dyes and, like the
gonococcus, is gram-negative. The involution forms found in cultures tend to
stain unevenly, of course, but even young cells may show the presence of
metachromatic granules when stained by Löffler's alkaline methylene blue and
other stains, and to a greater extent than the gonococcus. It may be noted that
no sure distinction between the meningococcus and the gonococcus can be
made on morphological grounds, and the identification of gonococci in gono-
coccal meningitis is dependent upon culture and differential fermentations.
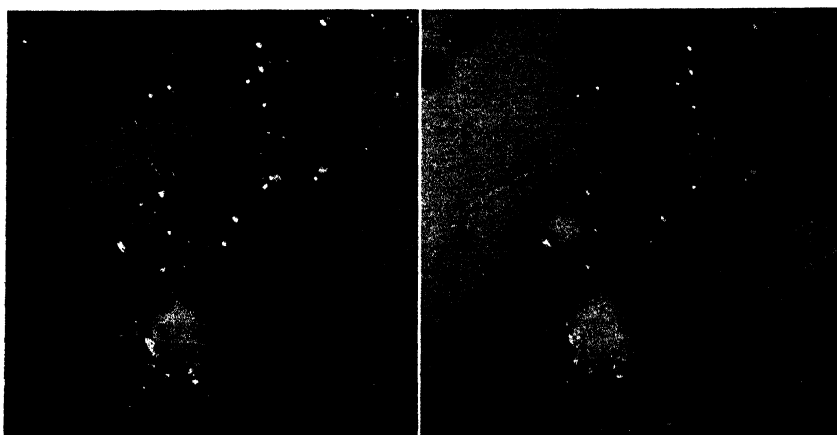
Fig. 61. The oxidase test for the identification of meningococcus colonies. Mixed cul-
ture on blood agar. Left, colonies of meningococci and contaminants before the application
of tetramethyl-*p*-phenylenediamine solution. Right, the same colonies after the application
of the reagent. Note that the meningococcus colonies show the development of color first
about the edges and there is slight discoloration of the medium. $\times$ 5.

Physiology. Strains of meningococci vary considerably in the ease with
which they may be cultivated; some strains will grow, although sparsely, on
nutrient and infusion media, but in general rich media containing serum or
whole blood are required. Infusion base chocolate agar or blood agar are the
most useful media. The hydrolyzed casein-starch medium of Mueller and Hin-
ton and Boor's tryptic digest medium will support growth of this bacterium.
According to Boor the meningococcus differs from the gonococcus in that it
does not require the addition of cystine to his medium. Frantz[27] has found
that stock cultures of the meningococcus, which are, however, less exacting
than recently isolated strains, will grow on an inorganic salt-glucose medium
containing glutamic acid and cystine, and others have reported that such strains
require only glutamic acid and glucose or lactate.[28] In isolating the meningo-
coccus it is essential that the culture medium be warm when inoculated and
kept warm until finally placed in the incubator. Growth is favored, espe-

[27] Frantz: Jour. Bact., 1942, 43:757.
[28] Grossowicz: Jour. Bact., 1945, 50:109.

cially in primary isolation, by incubation in an atmosphere containing 10 per cent carbon dioxide. The meningococcus will grow over a temperature range of 25° to 42° C. with an optimum at 37° C. Although sparse growth will occur under anaerobic conditions, the meningococcus is, for all practical purposes, an aerobe.

Continued cultivation on laboratory media results in more luxuriant growth, and the bacteria presumably become less nutritionally fastidious. Meningococcus cultures are difficult to keep, however, and tend to die out in stock culture. In most media these bacteria die within a few days if not transferred, but vitality may be preserved for several weeks in stab cultures in starch agar (1 per cent corn starch in nutrient agar) and are best kept in the incubator.

The relatively early appearance of involution forms in meningococcus cultures as well as their limited viability when not transplanted is, perhaps, attributable to their formation of an active autolysin, and in saline suspension in the incubator autolysis may take place within a few hours. The autolysin is heat-labile, being destroyed at 65° C in thirty minutes, and suspensions prepared for agglutination studies should be inactivated in this way.

The meningococcus, like the gonococcus, is a delicate microorganism and not highly resistant to deleterious influences. It is killed in a short time by drying and by exposure to dilute disinfectants. It is particularly sensitive to heat and cold and, unlike many bacteria, dies out within a few days at 0° C.

The meningococcus is not an active fermenter. Considerable quantities of acid, presumably lactic for the most part, are formed from glucose and maltose. The fermentation of maltose serves to distinguish the meningococcus from the gonococcus. Neither is this bacterium actively proteolytic, for coagulated serum is not liquefied.

Toxins. Meningococcus meningitis in man and that reproduced in experimental animals is usually accompanied by a profound toxemia. The meningococcus, however, appears to form no soluble toxin, though its cell substance is toxic to experimental animals when injected in relatively large amounts. Miller and his co-workers[29] have shown that the toxicity is heat-stable (100° C. for thirty minutes) and the rate of its destruction suggests that the endotoxin consists of two substances, one much more thermostable than the other. The relationship of the endotoxin to the "P" substance (see below) is not clear.

Variation. Rough and smooth colony types of the meningococcus have been described by Rake,[30] who found that recently isolated strains were generally smooth while old stock cultures were rough. Mucoid colonies were observed in a few instances. The change from smooth to rough was associated with a partial loss of immunological type specificity.

Classification. The meningococci are closely related to the gonococci, not only morphologically and physiologically but immunologically in that certain antigenic substances appear to be held in common as indicated previously.

The meningococci are not themselves immunologically homogeneous, and it was found by Dopter[31] that certain strains, culturally "typical" meningo-

[29] Miller *et al.*: Jour. Inf. Dis., 1943, 73:248.
[30] Rake: Jour. Exp. Med., 1933, 57:549, 561.
[31] Dopter: Compt. rend. Soc. biol., 1919, 66:1055.

cocci, did not agglutinate with antimeningococcus serum. These he designated "parameningococcus," a term that has since given rise to some confusion and may well be discarded. Gordon and Murray[32] distinguished four serological types, which they designated by Roman numerals. These types have been telescoped into two groups, Group I containing Types I and III which are very closely related, and Group II made up of Types II and IV. Type IV seems to have disappeared (it is doubtful whether the European Type IV and American Type IV were identical) in recent years and Group II and Type II are synonymous. There is considerable variation in the frequency of occurrence of the two groups, and in general Group I appears to be associated with the epidemic disease while Group II predominates in interepidemic years. Recently a new type, related to Type II but independent and homogeneous, has been found to be quite prevalent.[33] It has been designated Type IIa. The types of meningococci encountered at present are, then, Group I, Group (or Type) II, and Type IIa.

While it is not uncommon to encounter strains of meningococci which do not fall into these types, most strains may be assigned to one or another of them. In practice typing is complicated by the merging of types and the antigenic instability of meningococcus cultures. For routine identification agglutination or capsular swelling (*Quellung*) with polyvalent antiserum suffices.

Studies on the nature of the antigens of the meningococcus by Rake and Scherp[34] showed that three types of antigen are present. One is a polysaccharide common to all types of meningococci and found in the gonococcus also, which was designated "C" substance. A second fraction, likewise common to all meningococcus types and highly toxic to rabbits, is protein in nature and designated "P" substance. A third fraction is type-specific and in Types I and III is polysaccharide in nature and identical in the two types. The type-specific substance from Type II appears to be a polysaccharide-polypeptide complex.[35] Kabat, Kaiser and Sikorski[36] have prepared the polysaccharide from Type I.

While the routine typing of meningococci is probably not worth while, typing in connection with the preparation of therapeutic antisera is obviously of importance, and at the present time such antisera are generally polyvalent.

Pathogenicity for Man. The resistance of man to meningococcus infection is relatively high, and the incidence of healthy carriers is invariably considerably higher than that of cases of the disease. It is probable that predisposing factors play a large part in determining whether or not infection will occur; insufficient clothing, inadequate ventilation, exposure to inclement weather and fatigue very likely contribute in large measure to increasing susceptibility. In 1945, 7305 cases and 1539 deaths from meningococcus meningitis were reported by 44 states, rates of 6.2 and 1.3 per 100,000 population respectively; this is a decline from the record peak incidence in 1943 of 14.1 per 100,000.

The meningococcus is initially present in the nasopharynx and from there

[32] Gordon and Murray: Jour. Roy. Army Med. Corps, 1915, 25:411.
[33] Branham and Carlin: Proc. Soc. Exp. Biol. Med., 1942, 49:141.
[34] Rake and Scherp: Jour. Exp. Med., 1933, 58:341, 361.
[35] *Cf.* Menzel and Rake: Jour. Exp. Med., 1942, 75:437.
[36] Kabat, Kaiser and Sikorski: Jour. Exp. Med., 1944, 80:299.

gains access to the central nervous system. The route by which this occurs is uncertain; it is thought by some that the bacteria follow the perineural spaces of the olfactory nerves or set up a preliminary sinusitis and reach the brain either via the lymphatics or by direct extension through the bone. Others believe the meningococci reach the central nervous system via the blood stream through a preliminary bacteremia. While there is no definitive evidence concerning the means by which meningococci reach the central nervous system from the nasopharynx, the evidence appears to favor the hematogenous route. Occasionally the infection in the nasopharynx may extend into adjacent areas, giving rise to conjunctivitis, pneumonia, etc.

In the healthy carrier the infection remains confined to the nasopharynx and in this case is short-lived there and produces few or no symptoms. When the blood stream is invaded early in the disease hemorrhages usually occur in
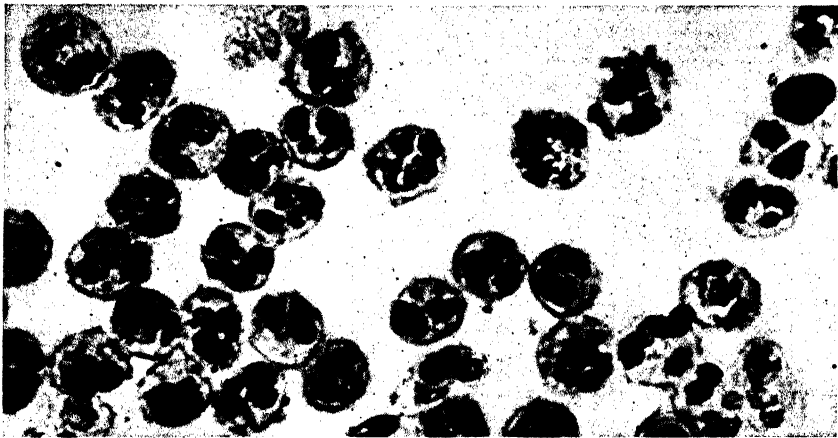
Fig. 62. Meningococci in spinal fluid, showing phagocytosis of the microorganisms. Gram stain; × 1050.

the skin and petechiae appear, especially on the wrists and ankles, or on any mucous or serous surfaces. These are apparent in twenty-four hours after invasion and fade in a few days. The rash is quite different from other purpuric rashes; the spots take geometric shapes and are highly irregular in size. Meningococci may be observed in smears of material taken from these lesions. Other symptoms include sudden onset, chills, fever, and meningeal symptoms such as headache, drowsiness, etc. Pain in the arms and legs is common.[37] The invasion of the blood stream may take the form of a fulminating meningococcemia (the Waterhouse-Friderichsen syndrome) with adrenal apoplexy as the immediate cause of death and massive bilateral hemorrhage of the adrenals as the outstanding pathology. This form of meningococcal disease is uncommon—little more than 200 cases have been reported—and occurs much more frequently in infants than in adults. Its sudden and violent character with rapid fatal termination has led to deaths due to this cause being classified as suspicious. The bacteremia may also take a more chronic form, and give rise to a purulent synovitis or meningococcal arthritis.

[37] For a general discussion see Strong: Amer. Jour. Med. Sci., 1943, 206:561.

Upon reaching the central nervous system, the meningococcus sets up a suppurative lesion of the meninges which involves the surface of the spinal cord together with the base and cortex of the brain. The microorganisms are invariably present in the spinal fluid, which may vary from a slight to a heavy turbidity. The bacteria are found, sometimes in great numbers, both free and within the leucocytes in smears of spinal fluid. The case fatality is variable but in any case high, and ranges from 35 per cent to 80 per cent and has been reduced to 16 per cent by improved methods of treatment, including chemotherapy.

There is some divergence of opinion regarding the nature and extent of sequelae to meningococcus meningitis, but these appear to include deafness,
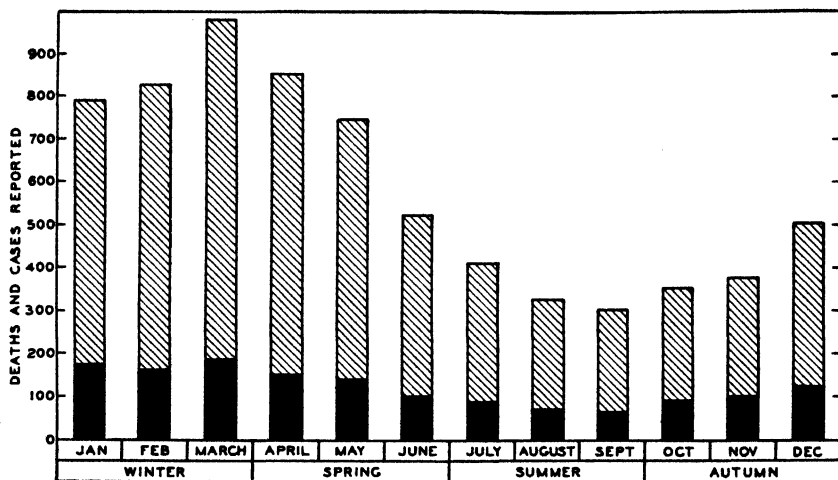
Fig. 63. The seasonal incidence of meningococcus meningitis. Averages of reported cases by months for the years 1939 to 1945 inclusive. Data from Supplements to Public Health Reports.

which is the most common, blindness, and pain and weakness of the neck, arms and legs. In the series of cases studied by Maddock[38] 7 to 8 per cent of the patients showed sequelae persisting for nine to thirty months after recovery.

Epidemiology. Like most of the respiratory infections, meningococcus meningitis is disseminated by direct contact and droplet infection through the secretions of the mouth, nose and throat. Infection is spread by patients and convalescents to a limited extent, but healthy carriers of meningococci are of primary importance. Some persons are temporary carriers, while others are chronic, discharging meningococci more or less continuously or in a sporadic fashion. Of a group of ten carriers studied at the Rockefeller Institute,[39] half were of the chronic type, carrying what was apparently the same strain of microorganism for over two years. Weekly examination might be negative for a period, in one case as long as four and one-half months, and then the same

[38] Maddock: Med. Res. Council (Great Britain) Monthly Bull. Ministry of Health and Emergency Pub. Health Lab. Service, 1943, 2:111.
[39] Rake: Jour. Exp. Med., 1934, 59:553.

type of meningococcus would appear. Miller and his co-workers[40] reported a continuous survey over a seventeen-month period of hospital staff and employees. The peak in carrier rate of 17 per cent was reached in April and May. Of 90 carrier strains isolated, 20 did not fall into the recognized types and 70 were typable. Of the latter group, 16 were Type I, 26 Type II and 7 Type IIa. It was of some interest that spouses of carriers were only occasionally carriers and then of a different type of meningococcus, suggesting a limited infectivity of the carrier strains.

The carrier rate is, of course, variable but is undoubtedly directly related to the incidence of clinical disease. Studies[41] during the first World War indicated that the normal carrier rate in some of the troops was 2 to 4 per cent but rose preceding an epidemic, and when it reached 20 to 30 per cent cases began to appear. As the epidemic developed the carrier rate continued to rise, sometimes to as high as 80 to 90 per cent. The increase in carrier rate appeared in
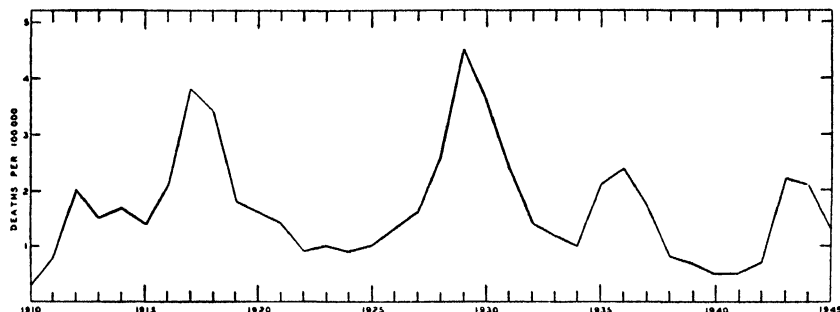
Fig. 64. The prevalence of meningococcus meningitis in the Registration Area since 1900 as indicated by the uncorrected death rates. From data compiled by the Bureau of the Census.

some instances to be associated with the spacing of the beds in barracks, occurring when the beds were within a few inches of one another; spacing of the beds 3 feet or more apart resulted in a reduction of the carrier rate from 30 per cent to less than 2 per cent. The general significance of these observations is uncertain, however, for in some outbreaks congestion of the population has appeared to be a minor factor,[42] in other instances the carrier rate may be 20 per cent or more without the occurrence of cases,[39] and sporadic cases occur with a low (5 per cent) carrier rate.

Epidemics of meningococcus meningitis are prone to occur in military populations, and epidemic cerebrospinal fever was, with influenza, one of the most important diseases among the troops in the first World War. The influence of predisposing factors causing an increased susceptibility is especially marked under conditions of military life; fatigue, exposure to inclement weather and similar factors very likely play a part in reducing the normal resistance of the raw recruit. This general reduced resistance, coupled with

[40] Miller, Beadenkopf, Peck and Robbins: Jour. Inf. Dis., 1944, 74:212.
[41] *Cf.* Med. Res. Council Spec. Rept. Ser. No. 50, 1920.
[42] As in the Detroit epidemic of 1928–29. *Cf.* Norton and Gordon: Jour. Prev. Med., 1930, 4:207.

the opportunities for the dissemination of the microorganisms provided by the close communal existence in barracks, undoubtedly is an important factor in the genesis of these epidemics. Outbreaks in the civilian population differ in that children (over three months) and adolescents are generally attacked; susceptibility appears to be greatest in children under ten years of age, distinctly less in adolescents, and remains at a low level thereafter.

Whether occurring in civilian or military populations, epidemics of meningococcus meningitis have a number of distinctive characteristics. The relatively high carrier rate that may prevail, coupled with the low morbidity, estimated at 0.01 to 0.3 per cent of the exposed population, is an expression of a high degree of normal resistance, while the high case fatality rate indicates the serious course of the infection once established. The relative insusceptibility of the general population also results in the spotty character of the spread of the disease; some groups escape which are closely associated with the focus of infection, while in others, apparently remote, outbreaks occur. Direct transmission from case to case is infrequently observed. Usually carriers constitute the link between cases. Further, epidemics frequently consist of a series of recurring outbreaks rather than the well marked single epidemic wave often observed to occur in other diseases. Since 1910 there have been four distinct epidemic periods (see Fig. 64), with a record incidence of 14.1 per 100,000 in 1943 as compared with 5.5 in 1936 and 7.5 in 1918. The last peak is not so apparent in the death rates with the sharp reduction in case fatality rate in recent years. The seasonal incidence is marked in temperate climates (see Fig. 63). Males are more frequently attacked than females, although this may be an expression of risk rather than sex differences in resistance. Racial differences in susceptibility are indicated by military experience in which the morbidity and mortality rates of the colored troops were twice that of the white.

Bacteriological Diagnosis of Meningococcus Infection. The meningococcus is present in considerable numbers in the spinal fluid in meningitis, and the finding of the characteristic gram-negative intracellular diplococci in stained smears of the sediment from centrifuged spinal fluid is sufficient to establish a provisional diagnosis. It may be cultured by the direct inoculation of spinal fluid sediment or nasopharyngeal swabs (in the detection of carriers) on infusion base chocolate agar or blood agar. Blood culture is useful early in the disease and in those cases which do not show meningeal symptoms. In any case it is important that the specimen not be allowed to cool below body temperature before inoculation of the warm medium. Like the gonococcus, the meningococcus is oxidase-positive and the oxidase test is especially useful for nasopharyngeal cultures. The isolated meningococci may be identified by fermentation tests and agglutination with polyvalent antiserum. Meningococci may be typed by agglutination, and in recent years the capsular swelling or *Quellung* reaction, described in the typing of pneumococci, has been used. Chicken antiserum is regarded as superior to rabbit antiserum by many workers. Typing is sometimes desirable in that a rise in the proportionate incidence of Type I usually precedes an epidemic of meningococcus meningitis, and dangerous carriers may be distinguished from relatively harmless ones.[48]

[48] *Cf.* Branham: Amer. Jour. Pub. Health, 1945, 35:233.

Pathogenicity for Lower Animals. The usual experimental animals are relatively resistant to the meningococcus upon intraperitoneal or intravenous inoculation. White mice are more susceptible than most other animals, and the injection of sufficient quantities of meningococci will result in death. Rabbits react in a similar fashion. Enormous numbers of bacteria must be injected, however, and there is some question as to whether an actual infection is set up; killed meningococci are as effective as the living cells, and it is likely that the observed result is essentially a toxemia.

Flexner[44] was able to infect rhesus monkeys by the intraspinal inoculation of large amounts of meningococcus cultures, and the disease appeared to be more acute than in man. Branham and her colleagues have been able to reproduce meningococcus meningitis in rabbits[45] and in guinea pigs[46] by the intracisternal injection of virulent meningococci. As in man, the experimental disease is both a local meningeal involvement with a purulent meningitis and a general toxemia. According to Branham it is easier to produce meningitis in guinea pigs than in rabbits. Similarly, Andrewes and Lush[47] have produced fatal meningo-encephalitis in mice by intracerebral inoculation though not by other routes. It is of interest to note that the developing chick embryo may be infected with meningococci; the twelve-day embryos develop a septicemia and hemorrhagic lesions simulating fulminating meningococcus septicemia[48] in man.

Immunity. Infection with the meningococcus leads to the development of demonstrable antibodies and patient's serum may agglutinate to a titer of 1:50. The development of agglutinins is irregular, or possibly appears so because of the antigenic instability of meningococci, and cannot be depended upon for diagnosis. Complement-fixing antibodies are produced also but too late in the disease (eight to fourteen days) to be of diagnostic utility.

The injection of large numbers of meningococci into horses induces the formation of agglutinins, opsonins and amboceptors, and the horse serum has a curative value. The therapeutic use of antiserum was placed upon a sound basis by the work of Flexner and his collaborators in 1907. A curative serum was prepared by injecting a horse first with gradually increasing doses of dead meningococci, then of living cocci, and finally of an autolysate. Injection into the spinal canal of this serum exerts, in many instances, a marked influence upon the course of the disease. The effect is due in part to an antitoxic action, in part to a stimulus to increased phagocytosis, and in part to a direct injurious effect upon the meningococci, for observations of the cerebrospinal fluid after injection show a remarkable destruction of the meningococci. The antiserum is apparently not particularly effective when given intravenously, but a combination of intravenous and intrathecal administration appears to be better than intrathecal injection alone. Flexner has reported[49] that in 1294 cases treated with serum the mortality was not quite 31 per cent, as compared with an average case fatality of 70 per cent in the pandemic which began in

[44] Flexner: Jour. Exp. Med., 1907, 9:142.
[45] Branham and Lillie: Pub. Health Repts., 47:2137.
[46] Branham, Lillie and Pabst: Pub. Health Repts., 1937, 52:1135.
[47] Andrewes and Lush: Jour. Path. Bact., 1941, 52:85.
[48] Buddingh and Polk: Jour. Exp. Med., 1939, 70:485, 499.
[49] Flexner: Jour. Exp. Med., 1913, 17:553.

1904 and had not wholly ended in 1913. In 199 cases in which antiserum was administered between the first and the third day of the disease the mortality was 18 per cent. Variation in the case fatality rates in various epidemics is wide, however, and it is difficult if not impossible to determine the relative effect of differences in the virulence of the meningococci, variation in the efficacy of the sera employed and differences due to early or late administration of the serum.

The use of polyvalent antiserum is, of course, a necessity, as pointed out earlier. Continued experience with antimeningococcus serum tends to emphasize some of the difficulties of practice and interpretation. Antiserum is standardized by the mouse protection test, using meningococci in mucin suspension as the challenge inoculum. In spite of the beneficial results frequently
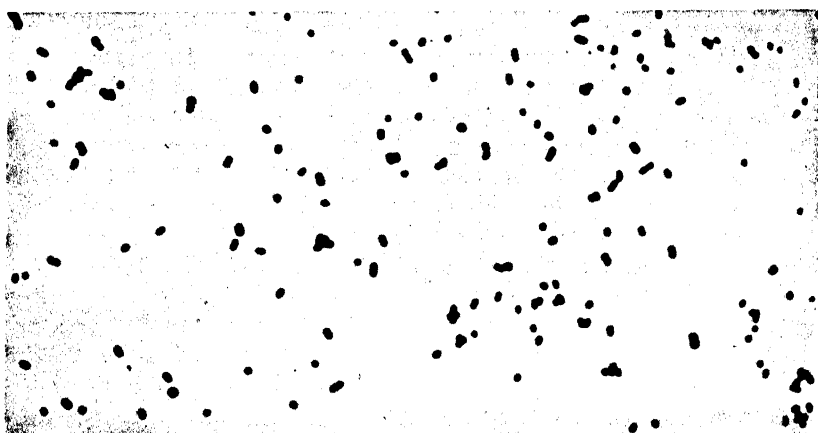
Fig. 65. *Neisseria catarrhalis.* Smear from a pure culture. Note the diplococci and elongated forms which have not yet divided. Fuchsin; × 1050.

reported from the use of antiserum, there are some cases and some epidemics in which the serum treatment seems of little avail.[50]

Prophylactic vaccination with suspensions of meningococci has been attempted by a number of workers, but the procedure is difficult to evaluate. For example, Maclean and Bevan[51] found no evidence of the efficacy of active immunization in their study of an epidemic in Cyprus, while Genevray[52] has reported highly encouraging results in Tonkin. In general there appears to be no consistent and unequivocal evidence of a significant degree of protection conferred by active immunization procedures.

OTHER GRAM-NEGATIVE DIPLOCOCCI

In addition to the gonococcus and the meningococcus, two other species of non-pigmented gram-negative diplococci are generally recognized.

Neisseria catarrhalis is found commonly in the nasopharynx of healthy individuals as well as of persons suffering from colds and other respiratory

[50] The treatment of meningococcus meningitis is discussed by Branham: Pub. Health Repts., 1938, 53:645.
[51] Maclean and Bevan: Proc. Roy. Soc. Med., 1939, 32:1551.
[52] Genevray: Rev. méd. franc. d'extreme-Orient, 1941, 19:143.

infections. The cells as a rule are somewhat smaller than those of the meningococcus. Growth occurs on ordinary nutrient agar much more readily than in the meningococcus, and the colonies are generally thicker and more opaque. Dextrose and other sugars are not fermented. Different strains vary in their pathogenicity for animals, but many strains are fully as pathogenic as meningococci for white mice. In man they appear at times to excite catarrhal inflammation and sometimes pneumonia and meningitis have been reported. They seem to have been conspicuous invaders in the 1918 influenza epidemic in some localities.

Neisseria sicca is a small gram-negative coccus found on the mucous membranes of the respiratory tract. It grows at room temperature as well as at 37° C., forms white, firm, dry, adherent colonies, and ferments sucrose, lactose and maltose. Although not ordinarily regarded as pathogenic, it has been found as apparently the causal agent in a case of kidney infection and has been found in the blood stream of patients ill with clinical endocarditis.

The Pigmented Forms. Gram-negative diplococci which form a pale greenish-yellow pigment often best observed by transmitted light may be found in the upper respiratory tract of man. Formerly regarded as non-pathogenic, one species, *N. flavescens,* has been described as occurring in the spinal fluid in cases of clinical meningitis, as indicated earlier. These pigmented forms are differentiated from one another on the basis of fermentations.

The Obligate Anaerobic Species. Three species of Neisseria are obligate anaerobes, *N. discoides, N. reniformis* and *N. orbiculata.* These bacteria are parasites of man and are found in the mouth, intestinal tract and genito-urinary tract, but are not of known pathogenicity. Obligate anaerobic forms related to Neisseria, but differing in that the cells occur in masses and sometimes in short chains rather than in pairs, are placed in the genus Veillonella. There are two species, *V. parvula* and *V. gazogenes,* formerly known as *Micrococcus parvula* and *Micrococcus gazogenes.* The former is weakly hemolytic and ferments glucose, while the latter is non-hemolytic and does not ferment carbohydrates. Both appear to be harmless parasites of man and are found primarily in the mouth and intestinal tract.

THE ENTERIC BACILLI: THE COLIFORM BACTERIA, FRIEDLÄNDER'S BACILLUS, AND PROTEUS

The gram-negative, non-spore-forming bacilli make up a large group of bacteria which includes intestinal commensals such as the colon bacilli and Proteus; the enteric pathogens such as the typhoid, paratyphoid and dysentery bacilli; certain saprophytic forms and plant pathogens; and, as more distant relatives, the hemophilic bacteria (Hemophilus); the so-called hemorrhagic septicemia group (Pasteurella); and the causative microorganisms of undulant fever (Brucella).

By far the largest of these subdivisions is that of the enteric bacilli whose habitat is the intestinal tract of man. Some are, for the most part, intestinal commensals with but feeble pathogenic powers while others are highly pathogenic, producing intestinal disease of greater or lesser severity. The cholera vibrio is sometimes considered as somewhat set apart from the enteric group proper because of its curvature; this would appear to be an overemphasis of a minor morphological difference for not only do the longer enteric bacilli often show a slight curvature, but after continued cultivation on laboratory media the cholera vibrios lose their curvature and become morphologically indistinguishable from the other enteric bacilli. Still other bacteria are closely related to the enteric bacilli, in fact sometimes cannot be distinguished from them with certainty, but are set apart by differences in habitat. Such are Friedländer's bacillus, a common inhabitant of the upper respiratory tract and the causative agent of a small proportion of pneumonias, and the plant pathogens of the genus Erwinia which produce soft rots of vegetables. Similarly, the members of the genera Proteus and Pseudomonas occur as free-living saprophytes as well as intestinal commensals of occasional pathological significance.

The taxonomic relationships of these forms is shown in the accompanying scheme in which the pathogenic enteric bacilli are included under the general head of Enterobacteriaceae together with Friedländer's bacillus, but Erwinia and Serratia, the latter chromogenic saprophytes of which *Bacterium prodigiosum* (*Serratia marcescens*) is the best known representative, are omitted. It may be noted that the formal speciation adopted by Bergey (1948) does not correspond too well with the kinds of bacteria included under the head of Eschericheae.

The enteric bacilli fall into certain natural groups on the basis of physiological, immunological and pathogenic characteristics. These are: the coliform group which includes *Bacterium coli* and *Bacterium aerogenes* together with intermediate and variant forms; the Salmonella group made up of the

typhoid and paratyphoid bacilli; the group of dysentery bacilli; and the cholera and paracholera vibrios. The differences between these groups, however, are differences of respective modal conditions of the differential characters, and at their peripheries the groups merge into one another to give a continuous series of intergrading types whose taxonomic positions and relationships have so far defied precise and satisfactory definition.[1] A useful primary differentiation can be made on the basis of the lactose fermentation which is roughly correlated with pathogenicity; the coliform bacteria ferment this sugar rapidly with the formation of acid and gas in twenty-four hours, while the bacteria of

RELATIONSHIPS OF THE ENTERIC BACILLI (ENTEROBACTERIACEAE)

Eschericheae..
- Escherichia ⎫ ....
- Aerobacter ⎭
  - coli type—*Bacterium (Escherichia) coli*
  - intermediate types—no species names
  - aerogenes type—*Bacterium (Aerobacter) aerogenes*
  - paracolon bacilli...... ⎰ coli type / intermediate types / aerogenes type
  - *Bacterium (Aerobacter) cloacae*
- Klebsiella............ *Bacterium friedländeri (Klebsiella pneumoniae)**

Proteae........ Proteus............
- *Proteus vulgaris*
- *Proteus mirabilis*
- *Proteus morgani*
- *Proteus rettgeri*

Salmonelleae..
- Salmonella..........
  - *Salmonella typhi*
  - *Salmonella paratyphi A*
  - *Salmonella typhi-murium* etc.
- Shigella............
  - *Shigella shigae*
  - *Shigella parashigae*
  - *Shigella flexneri*........Types I to VI
  - Boyd varieties........Types 170, P 288, D 1.
  - (*Shigella boydii*)
  - *Shigella ambigua (schmitzii)*
  - *Shigella alkalescens*
  - *Shigella sonnei*
  - *Shigella dispar*....... ⎰ var. *ceylonensis* / var. *madampensis*

The genera Erwinia and Serratia, plant pathogens and chromogenic (red) saprophytes respectively, are omitted here though classified with the Enterobacteriaceae.

* *Bact. friedländeri* is an upper respiratory rather than intestinal parasite but is closely related to the enteric bacilli.

the other groups, essentially pathogens, do not ferment it. The distinction is not absolute, of course, since both the paracolon bacilli and certain of the dysentery bacilli are slow lactose fermenters, but is sufficiently marked to have considerable practical value.

Similarly, the dysentery bacilli divide into two groups on the basis of the fermentation of mannitol and are anaerogenic, that is to say, do not produce gas from fermented carbohydrates. While the Salmonella group in general produces gaseous fermentations, the typhoid bacillus and *Salmonella gallinarum* are typically anaerogenic and anaerogenic strains of other Salmonellae

[1] Because of the lack of better general agreement than now exists regarding the taxonomy of these forms, the writer prefers to retain the generic name *Bacterium* for many of the enteric bacilli. As one worker has put it, a large part of the difficulty in classifying these forms arises from the fact that the "missing links" from a phylogenetic point of view are not missing.

BIOCHEMICAL REACTIONS OF THE ENTERIC BACILLI

Group	Species	Butt Russell's Slant	Dextrose	Lactose	Sucrose	Mannitol	Dulcitol	Sorbitol	Maltose	Rhamnose	Raffinose	Arabinose	Trehalose	Inositol	Xylose	Salicin	Milk	H₂S	Indol	Gelatin	d-Tartrate	l-Tartrate	m-Tartrate
Coliform Bacilli	*Bact. aerogenes*	G a	G	G	G	G	G/−	G	G	G	G	G	G	G	G	G	ac	−	−	−			
	Bact. coli var. communior	G a	G	G	G	G	G/−	G	G	G	G	G	G	−	G	−	ac	−	+	−			
	Bact. coli var. communis	G a	G	G	−	G	G/−	G	G	G	G/−	G	G	−	G/−	G	ac	−	+	−			
	Bact. cloacae	G ak	G	G	G	G	G/−	G	G	G	G	G	G	a	G	G	ac	−	−	+			
Dysentery Bacilli	*Sh. sonnei*	a or k	a	a(l)	a(l)	a	−	−	a(l)	a	a	a	a	−	−/a	a	a(l) ac	−	−	−			
	Sh. dispar var. ceylonensis	a or k	a	a(l)	a(l)	a	a	a	a(l)	a	a	a		−	a	a	a(l) ac	−	+	−			
	Sh. dispar var. madampensis	a or k	a	a(l)	a(l)	a	−	a(l)	a(l)	a		a		−	a	a	a(l) ac	−	+	−			
	Sh. shigae	a or k	a	−	−	−	−	−	−	a	−	a/−	−	−	−		ak	−	−	−			
	Sh. flexneri	a or k	a	−	a/−	a	−/a	a	a/−	a	a	a	a	−	a	a	ak	−	+/−	−			
	Sh. ambigua (Schmitz)	a or k	a	−	−	−	−/a	−	a	−		−			−		ak	−	+	−			
	Sh. alkalescens	a or k	a	−	a/−	a	a	a	a	a				−	a		k	−	+	−			
Salmonella	*S. typhi*	a ak	a	−	−	a	a(l)/−	a	a	−/a	a	a	a	−	a	−	ak	+	−	−	+	−	−
	S. gallinarium	a ak	a	−	−	a	a	−	a	a/−	−	a	a	a(l)	−	−	−	+	−	−	+	+	+
	S. pullorum	G ak	G	−	−	G	−	−	−	G	−	G	G	−	G	−	ak	+	−	−	−/−	+	−
	S. paratyphi A	G k	G/−	−	−	G	G	G	G/−	G	−	G	G	−	−	−	ak	−	−	−	−	−	−
	S. paratyphi B	G k	G	−	−	G	G	G	G	G/−	G	G	G	a	−	G	ak	++	−	−	+	+	+
	S. typhimurium	G ak	G	−	−	G	G	G	G	G/−	−	G	G	G/G	G	−	ak	++	−	−	+	−/+	+
	S. paratyphi C (Hirschfeld)	G ak	G	−	−	G	G	G	G	G	−	G	G	−	G	−	ak	++	−	−	+	+(l)	−
	S. cholerae-suis (American)	G ak	G	−	−	G	G/−	G	G	G	−	−	−	−	G	−	ak	+	−	−	+	−	+
	S. cholerae-suis var. kunzendorf	G ak	G	−	−	G	G/−	G	G	G	−	−	−	−	G	−	ak	++	−	−	+	−/+	+
	S. typhi-suis (Glässer-voldagsen)	G ak	a	−	−	a(l)/−	a	a	a	a	−	a	a	−	a	−	−	−	−	−	−	−	−
Others	*Alkaligenes fecalis*	− k	−	−	−	−	−	−	−	−	−	−	−	−	−	−	k	+	−	−			
	Proteus vulgaris	G k	G	−	G	−	−	−	−/G						G/−		kd	++	+	+			
	Proteus mirabilis	G k	G	−	G(l)	−	−	−	−								kd	++	−	+			
	Proteus morgani	G k	G	−	−	−	−	−	−	−	−				−/G	−	k	−	+	−			
	Proteus rettgeri	G k	a	−	a(l)	a	−	−	−						a/−		kd	−	+	−			

G—acid and gas; a—acid; G or a—most strains ferment; Ḡ or ā—most strains do not ferment; (l)—late; k—alkaline; ak—acid to alkaline; c—curd; d—digestion; +—utilisation of tartrate, formation of H₂S or indol, or liquefaction of gelatin; − —no action.

BIOCHEMICAL DIFFERENTIATION OF THE PATHOGENIC ENTERIC BACILLI

24 hour lactose fermentation— DC or SS medium

Coliform bacilli

No reaction *Alcaligenes fecalis*

Russell's double sugar medium

Acid butt, no gas, neut. alk. slant
{ *S. typhi* Shigella or Pseudomonas }

Acid and gas in butt, neut. or alk. slant
{ Proteus Salmonella‡ }

Blue-green soluble pigment

{ *S. typhi* Shigella }

Ps. pyocyaneus

Mannitol

{ *Sh. ambigua* *Sh. shigae* *Sh. parashigae* }

{ *S. typhi* *Sh. flexneri* *Sh. alkalescens* *Sh. sonnei* *Sh. dispar* }

Indol

Shigella ambigua (Schmitz)†
{ *Shigella shigae* *Shigella parashigae** }

Late lactose

{ *Shigella sonnei* *Shigella dispar* }

{ *S. typhi* *Sh. flexneri* *Sh. alkalescens* }

Sorbitol

Shigella dispar
Shigella sonnei†

Dulcitol (left)

Sh. dispar var. *ceylonensis**
Sh. dispar var. *madampensis**

Dulcitol (right)

{ *S. typhi* *Sh. alkalescens* }
Shigella flexneri†

Rhamnose

Sh. alkalescens‡
S. typhi†

Urease

Proteus

Salmonella‡

Mannitol

Pr. retigeri

{ *Pr. vulgaris* *Pr. mirabilis* *Pr. morgani* }

Maltose

Pr. vulgaris
{ *Pr. mirabilis* *Pr. morgani* }

Indol

Pr. morgan
Pr. mirabilis

* immunologically heterogeneous
† immunologically homogeneous, identify by serological methods
‡ subdivided into immunological species or types
NB S. *typhi* is more rapidly identified by initial culture or bismuth-sulfite agar and agglutination in typhoid antiserum

are occasionally met with. The general biochemical groups are indicated in the accompanying table of biochemical reactions.

The separation of the pathogenic enteric bacilli and the related bacteria which may be confused with them on a biochemical basis is of very considerable practical value but in general should be supplemented with, or can be cut short by, serological methods of identification. The specimen is usually cultured on a differential selective medium containing lactose and an indicator together with bile salts to inhibit the growth of contaminants. The biochemical differentiation of the lactose-negative bacteria may be carried out as illustrated in the accompanying dichotomous scheme.

The lactose-fermenting bacteria constitute the subject matter of the present chapter, and the non-lactose-fermenters will be discussed in the chapters immediately following.

BACTERIUM COLI

Bacterium coli (*Escherichia coli*) was described by Escherich in 1886 under the name of *Bacterium coli commune*. The original culture was isolated from the dejecta of a breast-fed infant, and cultures from this source were considered by Escherich to be especially typical. *Bact. coli* is, however, widely distributed, although "ubiquitous" only in the limited sense that it is universally found in the intestinal tract of man and many of the higher animals. It is especially abundant in the colon and is so characteristic an inhabitant of this region of the intestine as fully to deserve the name that has been given it. From fresh, healthy human feces it is often isolated in pure culture by the ordinary aerobic methods, although microscopic examination shows that other kinds of microorganisms are also present.

Morphology and Staining. The colon bacillus exhibits considerable variation in its morphology. The usual dimensions observed in stained preparations from cultures upon nutrient agar or gelatin range from 2 μ to 4 μ in length and from 0.4 μ to 0.7 μ in breadth. Very short, oval and coccuslike forms are not infrequently found and usually predominate when the bacillus is observed directly in the normal animal. The bacilli are occasionally observed in pairs or short chains. Some varieties are encapsulated, particularly those found in pathological conditions. Motility is variable, although the most typical strains are motile by peritrichous flagella. Spores are not formed.

Colonies upon nutrient gelatin are more consistent and characteristic in appearance than those upon agar media. They are opaque to partially translucent, smooth, moist and homogeneous in consistency, with entire to undulating edge, and exhibit the maple-leaf appearance common to many of the enteric bacteria (see below). Colonial morphology is somewhat variable upon nutrient agar. Typical colonies are opaque and grayish-white, and there may be a tendency to become a light yellowish brown upon continued incubation. Pigmented varieties are occasionally observed.[2] In certain differential media the colonies of *Bact. coli* may assume other characteristics typical under the special circumstances. Upon Endo's medium, for example, the colonies are, of course, red, but in addition take on a curious metallic sheen that is highly

[2] *Cf.* Gililland and Reese: Jour. Bact., 1943, 45:499.

characteristic when viewed by reflected light. Some varieties are β hemolytic on blood agar; such hemolytic strains occur with much greater frequency in pathological processes than in the normal intestine.

Bact. coli stains readily with the ordinary aniline dyes and is gram-negative. Flagella may be demonstrated by special staining methods.

Physiology. The colon bacillus is a facultative anaerobe, growing equally well under aerobic or completely anaerobic conditions. It grows luxuriantly upon the ordinary nutrient media and may be cultivated in synthetic solutions containing an ammonium salt and an organic source of carbon such as glucose. Growth occurs over a temperature range of 10° to 46° C., there is good growth from 20° to 40° C., and the optimum is 37° C.

Milk is curdled with an acid reaction, usually within forty-eight hours. Gelatin is not liquefied, but indol is produced in abundance by cultures in peptone water. Hydrogen sufide is produced in only very small amounts; cultures in the usual iron or lead acetate media are negative, but more sensitive methods give a positive reaction.

Various sugars are actively fermented with the production of acid and gas, including dextrose, lactose, maltose, arabinose, xylose, rhamnose and mannitol. The fermentation of other sugars, including sucrose, is variable, but the polysaccharides dextrin, starch and glycogen are not fermented. The gas produced is a mixture of carbon dioxide and hydrogen, generally in a ratio of 1:1. The greater part of the acid produced is lactic acid; smaller quantities of formic and acetic acids are formed, together with ethyl alcohol. Succinic acid is also found in variable but small amounts.

The colon bacillus is of ordinary resistance to deleterious influences, being, as a rule, neither as resistant as staphylococci nor as susceptible as the more delicate bacteria. Most strains are killed by exposure to 60° C. for thirty minutes, but occasionally more resistant varieties are encountered. In common with the other gram-negative bacteria, it is considerably less susceptible to the bacteriostatic action of dyes than are the gram-positive microorganisms, and selective media containing dyes are commonly used in the primary isolation of the enteric bacteria. The ability of the colon bacillus to grow in the presence of bile is likewise made use of in differential media (MacConkey's broth) in the bacteriological examination of water.

Variation. The existence of two colony types of *Bact. coli* has long been known, the one the flat, maple-leaf form, and the other a smaller, raised, round, moist type. The colon bacillus dissociates into rough and smooth colonial types, the S form giving rise to smooth, round, domed, shiny, translucent colonies, while the R colony type is characterized by an irregular, dull surface, jagged outline and opacity. It has been suggested that the R form is the more virulent.

Toxins. As in the case of many other bacteria, the cell substance is toxic to experimental animals upon parenteral inoculation. The specificity of the endotoxin has not been established. Vincent[3] has described two toxic substances formed by the colon bacillus, the one a heat-labile neurotropic toxin and the other a heat-stable enterotropic toxin. An enterotoxic substance

[3] Vincent: Compt. Rend. Acad. Sci., 1925, *180*:239, 407, 1083, 1624; see also Weinberg and Prevot: Compt. Rend. Soc. Biol., 1927, *97*:164.

that has been reported by other workers[4] is of interest in connection with the epidemiological implication of colon bacilli in outbreaks of food poisoning. Some strains of *coli* produce filterable hemolysin.

Pathogenicity for Man. The ability of the colon bacillus to set up pathologic processes in man is very slight. The microorganism is constantly present in the intestinal tract and, although excessive sugar fermentation with the liberation of irritant acids and gas may possibly be responsible for some cases of diarrhea, in general it does little harm. The term pathogenicity is, as has been pointed out elsewhere, a relative one, and *Bact. coli* may, on occasion, invade the body tissues and set up a focus of infection. The urinary tract is probably the most frequently invaded, and the majority of cases of cystitis are of *coli* etiology. The colon bacilli may also play a part in the formation of gallstones; the bacillus is frequently found in the core of gall-stones and, in culture, can precipitate cholesterol and other biliary constituents. The injection of colon bacilli into the healthy urinary bladder or gallbladder does not produce infection in experimental animals, but infection occurs if the bile duct or urethra is obstructed. Local infections such as abscesses, conjunctivitis and the like have been observed but are not common. *Bact. coli* septicemia is very rare, but may occur as an agonal invasion in acute infective processes. A hemorrhagic septicemia caused by *Bact. coli* sometimes occurs in newborn children and is known as Winckel's disease.

In general, however, *coli* infection is rare except in cystitis, and it is probable that the pathogenic powers of the colon bacillus have been exaggerated, particularly by the early writers who in many instances failed to differentiate *coli* from other members of the enteric group. It may be noted that the common occurrence of agonal or postmortem invasion of the body by the colon bacillus tends to diminish the value of evidence derived from finding this bacterium in the internal organs after death.

Pathogenicity for Lower Animals. *Bact. coli* is of low pathogenicity for laboratory animals. Two milliliters of a broth culture injected intraperitoneally will kill a guinea pig within a few days, and killed bacilli are very nearly as effective. Spontaneous infection of lower animals is not common. The diarrhea of young calves known as "scours" has been attributed to *Bact. coli* septicemia. The colon bacillus is also thought to be a factor in the causation of diarrhea of foals and young pigeons. Local infection may occur; in one series of 286 cases of bovine mastitis, 10 were apparently due to members of the colon-aerogenes group, of which 3 were typical *Bact. coli*.[5]

Varieties of Bacterium Coli. The above discussion has been one of "typical" *Bact. coli* and, although the description holds true in general, "atypical" strains are not infrequently found. These varieties may be separated, for pedagogical purposes, into two groups, one "fermentatively atypical," and the other, the strains intermediate between *Bact. coli* and *Bact. aerogenes*. The latter straddle the lines of demarcation drawn between the two species by various differential tests and will be discussed in a later section in that connection.

The Fermentative Varieties. These may, of course, be many, owing to

[4] Jordan and Burrows: Jour. Inf. Dis., 1935, 57:121.
[5] Gwatkin, LeGard and Hadwen: Canadian Jour. Comp. Med., 1938, 2:155.

the number of sugars whose fermentation by these bacteria is variable. A few of these are, however, well known and may be mentioned briefly. In regard to the sucrose fermentation, about half of the strains of *coli* isolated are able to ferment this disaccharide while the other half are not, and the two fermentative types have been given different names; the strains of *Bact. coli* that ferment sucrose are called *Bacterium coli communior*, while those that do not ferment sucrose are termed *Bacterium coli communis* (or *commune*).

Much less frequently strains of *Bact. coli* are isolated which do not immediately ferment lactose, *i.e.*, their colonies on Endo's medium are white. In the course of incubation, however, red papillae develop on the white colonies which, upon subculture, ferment lactose and breed true. Such strains have been known for many years and are termed *Bacterium coli mutabile*. This phenomenon is discussed elsewhere (p. 174) in connection with bacterial variation.

A fourth fermentative type which resembles *Bact. coli* in all particulars except that it ferments sugars with the production of acid and no gas is generally known as *Bacterium coli anaerogenes*. Although these names are commonly used they have no taxonomic standing and are a matter of convenience only.

The Paracolon Bacilli. Another fermentative variety whose relationship to the typical coliform bacteria is not clear is that which is characterized by late (five to fourteen days) lactose fermentation. In this respect these strains resemble the Sonne and *dispar* dysentery bacilli, and some strains resemble the typhoid and dysentery bacilli in that they are anaerogenic. They may be grouped into paracolon-coli, -intermediate, and -aerogenes strains.[6] They appear to be pathogenic to a minor degree, not only having been found in association with mild enteric infection, but also having produced laboratory infections. Perhaps the greatest difficulty in assessing the possible pathogenicity of the paracolon bacilli is that of identifying types and strains with precision. In a number of instances, however, immunologically identical or closely related strains have been found in outbreaks of diarrheal disease, and the close correspondence of the immunological character of the microorganism with the epidemiology of the disease is strongly suggestive of an etiologic relationship. They are perhaps to be regarded as transitional forms between the lactose-fermenters and the non-lactose-fermenters and possibly allied to the Sonne dysentery bacilli. The group is, however, a heterogeneous one and is not to be regarded as a well-defined type of enteric bacteria.

Bacterium cloacae, regarded by many as a distinct species, may be mentioned here, for it differs from *Bact. coli* in that indol formation is variable and gelatin is liquefied. The latter property serves to set *Bact. cloacae* off from the other members of the colon-aerogenes group, and it may be properly regarded as an intermediate form connecting the colon bacilli with the Proteus group, which do not ferment lactose but actively liquefy gelatin. *Bact. cloacae* has also been found to have some pathogenic properties.[7]

[6] See Stuart, Wheeler, Rustigan and Zimmerman: Jour. Bact., 1943, 45:101; Stuart and Rustigan: Amer. Jour. Pub. Health, 1943, 33:1323.
   [7] Caminita *et al.*: Pub. Health Repts., 1943, 58:1165.

BACTERIUM AEROGENES

Escherich originally described two types of gram-negative lactose-fermenting bacilli, the one *Bact. coli* and a second which was non-motile, somewhat shorter and plumper, and which clotted milk more rapidly. This second variety, first termed *Bacterium lactis aerogenes*, is now generally known as *Bacterium aerogenes* (*Aerobacter aerogenes*). *Bact. aerogenes* is commonly found in soured milk and, unlike *Bact. coli,* appears to live a saprophytic existence in nature on the surface of grains and similar places. Because of the apparent difference in the origin of these two kinds of lactose-fermenters, their differentiation has been a matter of great interest in connection with the use of *Bact. coli* as an indicator of fecal pollution (p. 253).

Because of the close resemblance of *Bact. coli* and *Bact. aerogenes,* the latter may be adequately described in terms of its differences from the former. The morphological differences are minor and variable and cannot be used
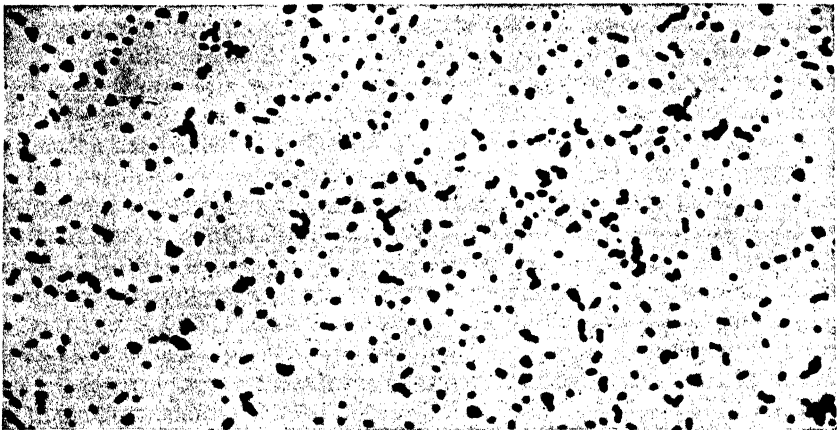
Fig. 66. *Bacterium aerogenes.* Smear from a pure culture. Note the coccobacillary form. Fuchsin; × 1050.

as differential characters. *Bact. aerogenes,* it may be noted, is often encapsulated, while the presence of capsules on *Bact. coli* is relatively infrequent. In general, the fermentative power of *Bact. aerogenes* is somewhat greater than that of *Bact. coli;* the ratio of carbon dioxide to hydrogen in the gas produced is about 2:1, milk is curdled more rapidly, and starch is fermented. Growth in gelatin is more luxuriant; in gelatin tubes a projecting "nail-head" growth is characteristically produced. Indol is not produced in peptone solution.

In conjunction with the above differences, three additional tests are commonly used in the differentiation of these bacteria:

(1) *The methyl red test* is no more than the determination of the *p*H of a dextrose broth culture after two to four days' incubation. *Bact. coli* produces and maintains a high acidity, and when the indicator is added it is red and *Bact. coli is said to be methyl red positive.* In *Bact. aerogenes* cultures, on the other hand, the hydrogen ion concentration is lower, the added indicator is yellow, and *Bact. aerogenes is said to be methyl red negative.*

(2) *The Voges-Proskauer reaction* is a qualitative test for the presence of acetylmethylcarbinol or, more properly, for diacetyl. In the fermentation of glucose *Bact. aerogenes* forms acetylmethylcarbinol along with organic acids and other products, while *Bact. coli* does not form this substance. The culture is grown in glucose-peptone broth for two to four days. When 5 ml. of 10 per cent potassium hydroxide solution is added a deep pink color develops on standing in cultures of *Bact. aerogenes* but not in cultures of *Bact. coli*. *Bact. aerogenes* is, then, Voges-Proskauer positive and *Bact. coli* is negative and there is a negative correlation between the Voges-Proskauer test and the methyl red test. The mechanism of the Voges-Proskauer test is as follows: upon standing in the presence of alkali the acetylmethylcarbinol present is oxidized to diacetyl, which in turn combines with an unknown constituent of the peptone to form the colored substance. Acetylmethylcarbinol is not formed during the early stages of fermentation by *Bact. aerogenes* but as a consequence of further decomposition of the initial products of fermentation; hence a two- to four-day culture must be used.

(3) The *citrate test* is dependent upon the ability of *Bact. aerogenes* to utilize sodium citrate as a sole source of carbon in a synthetic medium. A medium consisting of sodium ammonium phosphate, potassium phosphate, magnesium sulfate and sodium citrate is inoculated with the bacteria. *Bact. aerogenes* grows in this solution and is said to be citrate-positive; *Bact. coli* does not grow to any appreciable extent and is said to be citrate-negative.

The differential reactions of *Bact. coli* and *Bact. aerogenes* may be summarized in tabular form:

	$CO_2:H_2$	Indol	M. R.	V. P.	Citrate
Bact. coli...................	1:1	+	+	−	−
Bact. aerogenes..............	2:1	−	−	+	+

The four most commonly used tests, indol, methyl red, Voges-Proskauer and citrate, are frequently referred to by the mnemonic "Imvic" or "IMViC," which fixes them in order.[8] In this terminology typical *coli* is (+ + − −) and typical *aerogenes* (− − + +).

Other criteria have been used from time to time, including the fermentation of cellobiose, inositol, glycerol and other carbohydrates, the utilization of uric acid and the Eijkman test. The last consists in the fermentation of lactose at 45.5° ± 0.2° C., and gas formation in forty-eight hours is taken as positive. *Bact. coli* is positive to this test, while *Bact. aerogenes* is negative.

The Colon-Aerogenes Intermediates. "Typical" *Bact. coli* and "typical" *Bact. aerogenes* are, as indicated above, readily differentiated, but these bacteria represent extremes which are connected by a variety of intergrading forms. On the basis of the IMViC tests 16 combinations are possible, and all of these

[8] *Cf.* Parr: Amer. Jour. Pub. Health, 1936, 26:39. The relationships of the coliform bacteria are discussed at length in the review by Parr: Bact. Rev., 1939, 3:1. See also Vaughn and Levine: Jour. Bact., 1942, 44:487.

have been found.[9] The allocation of these intermediate forms to *coli* or *aerogenes* is, obviously, a difficult matter and probably not advisable. It is the practice of many to set up a "*coli* group," an "*aerogenes* group" and an "intermediate group" which may be referred to in the aggregate as the *coliform bacteria*. The existence of the intergrading types so lumped and the atypical strains of *coli* discussed earlier has interesting phylogenetic implications that cannot be considered here.

The Immunological Relationships of the Coliform Bacteria. The antigenic structure of the colon bacilli has been investigated by a number of workers and most intensively by Kauffmann.[10] There appear to be three kinds of antigens present, viz.:

(1) Heat-stable O antigens, of which 110 have been found. Of these, 25 occur with sufficient frequency for diagnostic use with most strains of coliform bacteria.
(2) Somatic surface antigens, designated as "envelope" antigens or K antigens. These function as "blocking antigens" in that their presence interferes with agglutination with O antisera. Three kinds of K antigens have been described:
  (a) Those which are designated L antigens are thermolabile and the O agglutinability of bacterial suspensions is restored by boiling. L antisera may be prepared by absorption of LO sera with the homologous O antigen, *viz.*, boiled bacteria. The colonies of strains containing L antigens are somewhat more opaque than those which do not contain them. About 24 L antigens have been described.
  (b) The component of the K antigen designated the A antigen is present in encapsulated coliform bacilli, is a specific polysaccharide, and bacteria containing it give a *Quellung* reaction in antiserum. It differs from the L antigen in that it is thermostable. So-called N variants lacking the antigen are found in translucent areas at the edge of the large, dense and relatively opaque colony. About 20 A antigens have been found.
  (c) An antigenic component of the K antigen complex, designated the B antigen, is thermolabile but differs from the L antigen in that the heated antigen can absorb antibody though heated suspensions will not agglutinate in monospecific B antiserum. The B antigen appears to be relatively rare.
(3) The flagellar or H antigens of the coliform bacilli are often poorly developed. Some 22 components have been found of which 20 are used for purposes of identification.

Kauffmann has developed a serological classification of coliform bacilli, largely *Bact. coli*, based on the distribution of O, K and H antigens. In general, about 80 per cent of strains having K antigens contain L antigens, and the other 20 per cent contain A antigen or B antigen. Strains containing K antigen appear in a general way to be the more toxic and more resistant to phagocytosis and the bactericidal action of antibody, and are found more frequently in pathological material than in feces. Some of the coliform bacteria are immunologically related to some of the other gram-negative bacilli, such as the plant pathogens, Friedländer's bacillus and Salmonella, while others appear to be related to certain of the pneumococcus types.

Wallick and Stuart[11] have made the interesting observation that while most of the colon bacilli harbored by a given individual were immunologically identical, or nearly so, individuals showed a continuous succession of types, each predominating for a few weeks or months and then being replaced by a

---

[9] Sanborn: Jour. Bact., 1944, *48*:211.
[10] See the review by Kauffmann: Jour. Immunol., 1947, *57*:71.
[11] Wallick and Stuart: Jour. Bact., 1943, *45*:121.

fresh type. It was noted also that immunologically identical types were some-times biochemically different.

The Ecology of the Coliform Bacteria. The distribution of the coli-form bacteria in nature is a matter of some importance in connection with the use of these organisms as indicators of fecal pollution and has been studied by a number of workers. The results of these studies are most conveniently expressed in the accompanying tabular form modified from Griffin and Stuart.[12] Although the percentages given there may not be regarded as an accurate expression of the distribution of the coliform bacteria in nature, it will be

PERCENTAGE DISTRIBUTION OF COLIFORM TYPES*

Source	Coli	Aer.	Int.	Irr.	Total
Milk....................	28.8	49.5	20.3	1.4	2224
Water...................	51.6	28.6	18.5	1.3	9496
Soil....................	23.8	54.3	18.8	3.1	1330
Grains..................	17.9	73.8	7.3	1.0	587
Feces...................	87.9	5.2	6.8	0.1	3974

* As compiled from various authors. In many instances the strains studied were not isolated at random; the percentages are, therefore, weighted, and great significance cannot be attached to them.

clear that no strict lines of demarcation regarding habitat may be drawn. Aerogenes and intermediate types are found in the intestinal tract in appre-ciable proportions, but it is probable that *coli* found in water, soil and elsewhere in nature is present as contamination and is *always* fecal in origin.

FRIEDLÄNDER'S BACILLUS

A heavily capsulated bacterium, closely related to the coliform bacilli,[13] was described by Friedländer in 1883 as the causative agent of pneumonia. As indicated in a previous chapter, the pneumococcus is the causative bac-terium in the great majority of lobar pneumonias, but the bacillus described by Friedländer is also responsible for a small proportion of pneumonias in man. The bacterium has been variously known as the *pneumobacillus*, *Bac-terium pneumoniae*, *Bacterium friedländeri*, *Bacillus mucosus capsulatus*, *Encapsulatus pneumoniae* and *Klebsiella pneumoniae*.

Morphology and Staining. Friedländer's bacillus is a short, thick oval rod 1 to 2 μ long and 0.5 to 0.8 μ thick, arranged singly and in pairs end to end. The diplobacillus arrangement is common in the body. Some strains are pleomorphic in culture, showing filaments and other forms, but these are not usual. These bacteria are non-motile and non-spore-forming. A heavy capsule

---

[12] Griffin and Stuart: Jour. Bact., 1940, 40:83.
[13] See the comparative study by Osterman and Rettger: Jour. Bact., 1941, 42:699.

is present on the bacilli in the body and is particularly evident in cultures on the richer media such as blood agar or media containing sugar.

A slimy growth is produced on artificial media, and the colonies are round, amorphous, raised with a glistening surface and mucoid in consistency. There is no hemolysis on blood agar.

The Friedländer bacilli stain readily with the usual aniline dyes, and are gram-negative. The capsule may be demonstrated in stained smears by special methods.

Physiology. These bacteria are facultative anaerobes whose optimum temperature is 37° C. and temperature growth range from 12° to 43° C. Nutritive requirements are exceedingly simple; these microorganisms grow luxuriantly upon the ordinary nutrient media and in simple synthetic solutions. In the writer's laboratory, representatives of all the types of Friedländer's bacillus have been found to grow well in the presence of an ammonium salt and simple carbon compounds such as acetate and, in one instance, formate.

Fig. 67. Friedländer's pneumobacillus showing the capsules; blood-agar culture; fixed with methyl alcohol and stained with carbol gentian violet (Wherry).

Fermentation reactions are highly variable from strain to strain, but in general a number of sugars are fermented. The lactose fermentation is variable. Gelatin is not liquefied and indol production is variable. In view of the relation of these bacteria to the coliform group it may be noted that the methyl red and citrate tests are positive, while the Voges-Proskauer test is negative.

The Friedländer bacilli are killed by exposure to 55° C. for thirty minutes but are unusually resistant to drying and are said to survive for a period of months. They are of average resistance to the usual germicidal chemicals.

Variation. These bacteria dissociate as rough and smooth forms. The commonly observed colony type is regarded as the smooth form although analogous to the "mucoid" phase observed in some other bacteria. As in the case of the pneumococcus, the S-R transformation is associated with the loss of the capsule and with a loss of immunological type specificity. The smooth form is virulent and the rough form avirulent.

Classification. Although an inhabitant of the upper respiratory rather than intestinal tract, Friedländer's bacillus is closely related to the coliform bacteria, especially *Bact. aerogenes.* In the Bergey (1948) classification it is

grouped with *Bact. coli* and *Bact. aerogenes* under the Eschericheae as *Klebsiella pneumoniae*. This bacterium is, however, exceedingly difficult to differentiate from the mucoid phases of the coliform bacteria and it is doubtful as to whether its individuality is such as to justify its status as the sole representative of a separate genus. It seems preferable, therefore, to include it in the same genus as the coliform bacteria as *Bacterium friedländeri*.

Types. In spite of a number of attempts it has not been possible to make use of fermentations or other biochemical reactions in subdividing *Bact. friedländeri*. Through the work of Julianelle,[14] however, it has been found that these bacteria may be divided into sharply defined immunological types. Analogous to the situation in the pneumococci, two types of antigen are present.
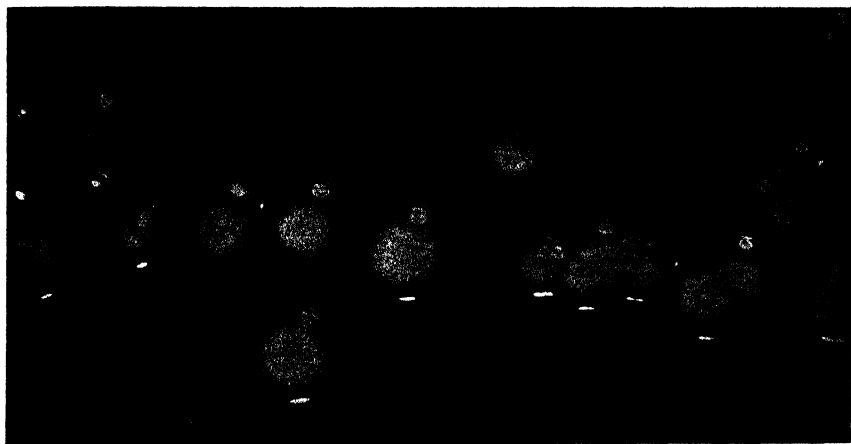
Fig. 68. Colonies of Friedländer's bacillus on blood agar. Note the large size and mucoid appearance. × 3.

The one, apparently nucleoprotein in nature, is species-specific, and the other, a polysaccharide present in the capsule, is type-specific. Julianelle has differentiated three serological types, Types A, B and C, to which the majority of strains belong, and a Group X for the remainder. Type B is immunologically similar to, although not identical with, Type 2 pneumococcus. The relative proportion of these types found in nature is indicated by Julianelle's study of 80 strains; 42 belonged to Type A, 12 to Type B, 7 to Type C and 19 to Group X. The Type A strains were largely from human sources and the Type B strains from lower animals. He has also reported that in Friendländer's bacillus pneumonias, Type A was found in 64 per cent, Type B in 14 per cent, Type C in 7 per cent and Group X in 15 per cent.

Pathogenicity. Microorganisms of this group are not infrequently found associated with various upper respiratory infections in man, though it is likely that in most instances they are secondary invaders. They are commonly present in the nasopharynx of persons suffering from chronic sinusitis or chronic lung infections such as bronchiectasis. Pneumonia due to Friendländer's bacillus is rare and makes up less than 1 per cent of all pneumonia, but the case-fatality

[14] Julianelle: Jour. Exp. Med., 1926, 44:113, 683, 735; *ibid.*, 1930, 52:539; Ann. Int. Med., 1941, 51:190.

rate is high, 90 per cent or more.[15] Members of this group have also been associated with suppurative conditions in various parts of the body, such as liver abscess, and, rarely, have been found to invade the blood stream and give rise to septicemia.

Friedländer's bacillus infection may also occur spontaneously in lower animals and has been incriminated in a spontaneous respiratory epidemic in mice, a paralytic disease of moose and metritis of mares. Experimentally the virulence of Julianelle's types differs; mice are highly susceptible to Types A and B, rabbits and guinea pigs are somewhat more resistant. Type C is relatively avirulent.

Related Bacteria. Two other bacteria, closely related to Friedländer's bacillus, have been considered by some to be associated with specific infections. A bacillus found in a fetid catarrhal condition of the nose known as ozena closely resembles *Bact. friedländeri,* as does the so-called rhinoscleroma bacillus found in the granulomatous lesions of a rare disease of the upper respiratory tract termed rhinoscleroma. Julianelle has found that the latter microorganism is identical with Type C Friedländer's bacillus,[16] but *Bact. ozenae* is immunologically distinct from both *Bact. friedländeri* and the rhinoscleroma bacillus.

PROTEUS

These microorganisms were originally described by Hauser as an independent genus containing three species—*Pr. vulgaris, Pr. mirabilis* and *Pr. zenkeri.* It is now generally agreed that the last is not closely related to the typical members of the group (it is gram-positive), and it is now placed in a separate genus as *Kurthia zenkeri.* As indicated earlier, Proteus is included under the Enterobacteriaceae and is related to but nevertheless distinct from the other enteric bacilli. It is found with some frequency in normal feces and often increases proportionately during or immediately after attacks of diarrheal disease caused by other organisms. It is one of the most common bacteria in soil and water containing decaying organic matter of animal origin and usually occurs in large numbers in sewage; it is perhaps to be identified with the "bacterium of putrefaction" or *Bacterium termo* of early writers. Like the enteric pathogens, it does not ferment lactose and it resembles them in its growth on differential and selective media. It may be confused with Salmonella because of its motility and gas formation during carbohydrate fermentation, but is distinguished by its ability to hydrolyze urea.

Morphology and Staining. In general, these bacteria appear as straight or slightly curved rods 1 to 2.5 μ in length and 0.4 to 0.6 μ in breadth, frequently in end-to-end pairs and short chains. Ovoid forms are common, however, and long, curved, filamentous cells predominate in actively swarming cultures. Proteus is actively motile by peritrichous flagella and forms neither capsules nor spores.

The phenomenon of "swarming" exhibited by these bacilli is a consequence of their active motility. On the surface of agar media the colonies do not remain compact and discrete; the growth spreads rapidly over the entire

[15] See the discussion by Hyde and Hyde: Amer. Jour. Med. Sci., 1943, 205:660.
[16] Morris and Julianelle: Jour. Inf. Dis., 1934, 55:150; Jour. Bact., 1935, 30:535.

surface available as a thin, scarcely visible bluish film. Microscopic observation shows that the bacilli break away from the edge of the growth and migrate or "swarm" over the surface of the medium, thus giving rise to the thin film of growth. This property is a source of considerable inconvenience in the isolation of bacteria other than Proteus from mixed cultures in which it is present; the inclusion of 0.01 per cent sodium azide inhibits the growth of this and other gram-negative bacilli but allows the growth of streptococci.

These bacilli stain readily with the usual aniline dyes and are gram-negative.

Physiology. The nutritive requirements of Proteus are simple, and the bacilli grow readily upon the ordinary laboratory media. They may be culti-vated in synthetic solutions containing ammonium lactate, but nicotinic acid must be supplied.[17] The optimum temperature for growth is 30° to 37° C.,
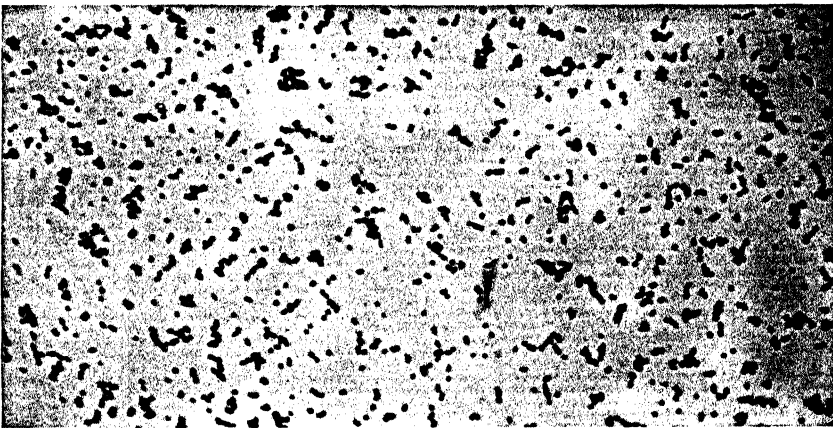
Fig. 69. *Proteus vulgaris.* Smear from a pure culture. Note the coccobacillary form. The occasional occurrence of paired cells is a result of active multiplication. Fuchsin; × 1050.

though good growth occurs at 20° C. These bacteria are facultative anaerobes, but anaerobic growth is generally very poor.

Gelatin is liquefied more or less rapidly, often with a characteristic colony formation with radiating filaments which wander far off into the surrounding medium. The typical Proteus colonies are best formed when the gelatin is soft, as happens when it is kept at a temperature not far below the melting point or is made up with 5 instead of 10 per cent gelatin. Dextrose is fermented with acid and gas production; sucrose and maltose are fermented by some, but not by all, strains; lactose, raffinose and mannitol are never fermented by the typical *Pr. vulgaris.* The Voges-Proskauer reaction is negative and the methyl-red test positive. Nitrates are reduced. Milk is at first rendered slightly acid, then curdled with alkaline reaction (in about three days), and more or less slowly peptonized. While species of Proteus other than *Pr. morgani* are actively proteolytic, these bacteria apparently do not play an important part in the anaerobic decomposition of proteins or putrefaction as was once sup-posed. Under aerobic conditions proteolysis occurs rapidly, *i.e.,* decay or non-putrefactive proteolysis.

[17] Fildes: Brit. Jour. Exp. Path., 1938, *19*:239.

Moltke[18] found that production of hydrogen sulfide and the decomposition of urea distinguish Proteus from all other gram-negative gelatin-liquefying bacilli. In a study of 194 strains he observed a definite division on the basis of maltose fermentation; 37 fermented maltose and 157 did not. All but one of the maltose-positive strains produced indol and none of the maltose-negative did. There were other correlated qualities. At the present time the bacteria comprising the genus Proteus are classified entirely on a biochemical basis. As noted above, the group is distinguished by its ability to hydrolyze urea; the liquefaction of gelatin is no longer regarded as a significant character. Four species are recognized, *Proteus vulgaris, Proteus mirabilis, Proteus rettgeri* and *Proteus morgani;* the last is known in the earlier literature as Morgan's bacillus (see below). These are distinguished from one another on the basis of the fermentation of mannitol, maltose and sucrose and the formation of indol.

Antigenic Structure. Proteus contains both H and O antigens (p. 301) when motile, and the non-motile, non-swarming strains contain only O

Fig. 70. *Proteus vulgaris* colony on blood agar. Note the swarming exhibited as successive waves of growth (Dack).

antigen. The species of Proteus are immunologically heterogeneous but common antigens are frequently encountered and an immunological classification of these bacteria has not been practical. Certain Proteus strains are agglutinated by the serum of patients having typhus fever. These so-called X strains contain an antigen common to the typhus rickettsiae and the agglutination of these bacilli (the Weil-Felix reaction) is of diagnostic value in typhus fever (p. 820). In the Proteus strains the antigen is a part of the O antigen and its specificity is determined by an alkali-stable carbohydrate haptene which is also found in the *Rickettsia prowazeki.*[19] The best known of the X strains is X-19. The X strains, it may be noted, frequently ferment maltose.

Pathogenicity. Proteus, both in mixed and pure cultures, has been found to be associated with a variety of pathological conditions. Infections of the eye and ear, pleuritis and peritonitis and suppurative abscesses in many parts of the body are among the many instances in which the pathogenic power of this bacterium in pure culture can hardly be doubted. As a producer of cystitis it probably ranks next to *Bacterium coli*. Besides its independent

[18] Moltke: *Contribution to the Characterization and Systematic Classification of Bact. proteus vulgaris (Hauser).* Copenhagen. 1927.

[19] *Cf.* White: Brit. Jour. Exp. Path., 1933, *14*:145; Castaneda: Jour. Exp. Med., 1934, 60:119: *ibid.,* 1935, 62:289.

pathogenicity, it is found so commonly associated with other microorganisms in purulent war wounds and similar processes that its activity as an accomplice is probably second only to that of the cocci.

In certain affections of the digestive tract Proteus has been frequently held to be the responsible agent. In diarrheic stools, especially those of infants, it has often been found in large numbers, and is regarded by many as a cause of infant diarrhea.[20] The real relation of Proteus to intestinal infection is, however, still obscure.

Certain food-poisoning epidemics have been ascribed to Proteus. These cases have been collected and subjected to a critical analysis by Bengtson,[21] who concludes that a definite proof of the causal relationship between the bacterium isolated and the illness is lacking in all instances. Further evidence is needed to establish the role of Proteus in food poisoning.

Animal inoculation shows that a great range of virulence exists among the Proteus cultures that have been tested. Freshly isolated strains from pathological sources may produce definite lesions, including abscesses, enlargement of the spleen and a diarrheic condition. One strain from a case of peritonitis has been reported which killed mice in amounts of 0.005 ml. of broth culture. The etiologic agent of "red-leg" disease of frogs, "ulcer disease" of brook trout, and "red sore" of pike has been found to be a bacillus closely related to the Proteus group and it has been classified as *Proteus hydrophilus.*[22] The cell substance of these bacteria is toxic on parenteral injection and, as in the case of the enteric forms, appears to be a glucolipid. No difference in toxicity is apparent between strains of pathogenic and non-pathogenic origin.

Morgan's Bacillus. This bacillus was isolated by Morgan[23] from the stools of infants suffering from summer diarrhea. Although it was first designated as "Morgan's No. 1," the other varieties of "Morgan's bacilli" have long since faded out of general interest and recognition, so that the name Morgan bacillus is usually applied to "Morgan's No. 1." In its cultural characteristics Morgan's bacillus resembles the coliform bacteria. Although it does not liquefy gelatin, it is closely related to the Proteus group, and it is therefore classified as *Pr. morgani.*

This bacillus appears to have played a part in a number of outbreaks of summer diarrhea of infants and has been isolated from paratyphoid-like fevers. It has been found to give rise to spontaneous epidemics of enteritis in mice. Injected intraperitoneally into mice, Morgan's bacillus produces a rapidly fatal infection.

[20] *Cf.* Neter and Farrar: Amer. Jour. Digest. Dis., 1943, 10:344.
[21] Bengtson: Jour. Inf. Dis., 1919, 24:428.
[22] Kulp and Borden: Jour. Bact., 1942, 44:673; Reed and Toner: Canadian Jour. Res., Sec. C, 1942, 20:161.
[23] Morgan: Brit. Med. Jour., 1906, i:908.

THE ENTERIC BACILLI: THE SALMONELLA GROUP[1]

The first member of this large group was described in 1888 by Gärtner, who isolated it from diseased beef responsible for an outbreak of gastro-enteritis, and named it *Bacillus enteritidis*. Similar bacteria have been found frequently in foods epidemiologically implicated in outbreaks of food poisoning, others are responsible for outbreaks of disease in rats, mice and other rodents, others are found in the paratyphoid fevers in man, and still others are responsible for certain poultry diseases.[1] These bacteria are parasitic and, although widely distributed in nature, do not maintain a saprophytic existence.

Morphology and Staining. These bacilli are gram-negative rods closely resembling and indistinguishable from the coliform bacteria. They stain readily with the usual dyes such as methylene blue and carbol fuchsin. No particular arrangement of the cells is apparent on microscopic examination. All species except *S. pullorum* and *S. gallinarum* are actively motile by means of peritrichous flagella. No capsules are apparent and spores are not formed.

Physiology. The bacteria of this group have simple nutritional require-ments, growing readily on the usual nutrient media. In synthetic media an ammonium salt and glucose, pyruvate, lactate, etc. are adequate sources of nitrogen and carbon, and the great majority of strains do not require bacterial vitamins or amino acids.[2] The optimum temperature is 37° C. but growth occurs at a reasonable rate at room temperature. They are facultative anaerobes, growing equally well under either aerobic or anaerobic conditions, and some species develop relatively strong reducing intensities.

The group is characterized biochemically by failure to ferment lactose or salicin, and inability to liquefy gelatin or produce indol. There are a very few exceptions to the two last; *S. eastbourne* and some strains of *S. enteritidis* and *S. panama* produce indol, and *S. dar-es-salaam* liquefies gelatin. The evolution of gas commonly accompanies the fermentation of sugars though anaerogenic strains of *S. enteritidis*, *S. typhi-murium* and *paratyphi* C have been reported.

In the past considerable significance has been attached to the differential biochemical reactions of these bacilli and they have been separated on this basis. In recent years, however, the antigenic structure of these bacteria has been emphasized and antigenic analysis has been carried further with this group than with any other bacteria. The differential value of sugar fermenta-tions and other biochemical reactions remains of primary importance neverthe-less. The biochemical reactions of some of the more frequently encountered Salmonella species are given in the accompanying table (p. 441).

[1] For a comprehensive discussion of these bacteria see Kauffmann: *The Bacteriology of the Salmonella Group*. Munksgaard, Copenhagen. 1941.

[2] Lederberg: Arch Biochem., 1947, *13*:287.

Toxins. No soluble toxin is formed by the bacteria of the Salmonella group, but, as is the case of many other bacteria, their cell substance is toxic upon parenteral inoculation. This toxic quality of two species, S. *enteritidis* and S. *aertrycke*, has been investigated and found to be a property of contained glucolipids (p. 205 and p. 284) which have immunological individuality (somatic antigens) and hence are, in a sense, specific. Pharmacologically, however, they are not specific, and their activity does not differ to a marked degree from that of the cell substance of other bacteria.[3]

The Immunological Differentiation of Salmonella Bacilli. The techniques of antigenic analysis have been developed through the efforts of White[4] in the study of the Salmonella group, and the antigenic composition of bacteria of this group is, perhaps, better known than that of any other
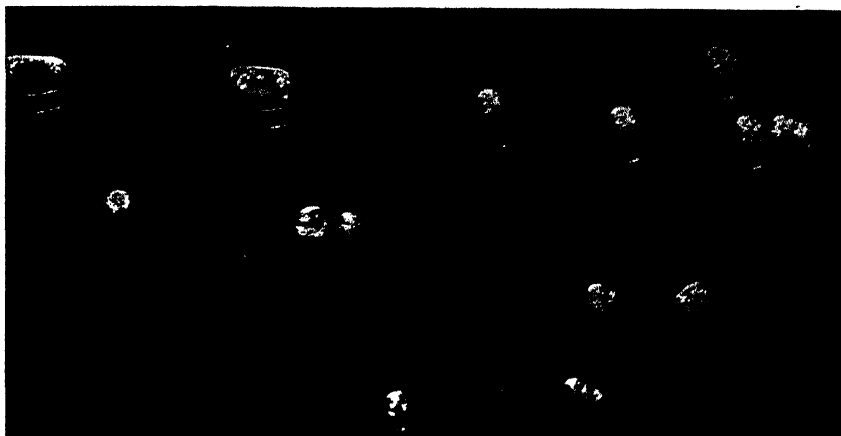
Fig. 71. Colonies of *Salmonella typhi-murium* (*aertrycke*) on nutrient agar. Twenty-four-hour culture. × 3.

bacteria. Many members of the group are immunologically complex; whether because they are innately so or because they are better known in this respect than most other bacteria is difficult to say.

Flagellar and Somatic Antigens. Two types of antigens are present in the bacteria of the Salmonella group, one associated with the cell substance and the other with the flagella. Early noted by Smith and Reagh,[5] the first was termed *somatic antigen* and the second *flagellar antigen.* These types were later observed by Weil and Felix,[6] who designated them O and H antigens respectively (p. 301). The O antigens are arbitrarily designated by Roman numerals. Two species of Salmonella, S. *paratyphi* C and S. *ballerup*, also contain Vi antigen (p. 451); this antigen is heat-stable and in this and certain other respects appears to be closely related to the O antigens.

These two types of antigen, each of which may be, and frequently is, represented in a single bacterial strain by more than one component, differ from

[3] See Cameron, Delafield and Wilson: Jour. Path. Bact., 1940, 51:223.
[4] White: Med. Res. Council Spec. Rept. Series No. 103, 1926.
[5] Smith and Reagh: Jour. Med. Res., 1903, 10:89.
[6] Weil and Felix: Ztschr. f. Immunitätsforsch., 1920, 29:24.

THE SALMONELLA TYPES (SPECIES)

Immunologic Types		Antigenic Formulae*		
			Flagellar Antigens	
		Somatic Antigens		
Group	Species		Phase 1	Phase 2
A	*S. paratyphi A*	(I), II, XII	a	
B	*S. paratyphi B*	(I), IV, (V)	b	(1, 2)
	S. abony	(I), IV, V	b	e, n, x
	S. typhi-murium	(I), IV, (V)	(i)	1, 2, 3
	S. stanley	IV, V	d	1, 2
	S. heidelberg	IV, V	r	1, 2, 3
	S. chester	IV, (V)	e, h	e, n, x
	S. san diego	IV, (V)	e, h	e, n, z_{15}
	S. salinatis	IV	d, e, h	d, e, n, z_{15}
	S. saint paul	I, IV, V	e, h	1, 2, 3
	S. zagreb	IV, V	e, h	1, 2
	S. reading	IV	e, h	1, 5
	S. kaposvar	IV, V	e, (h)	1, 5
	S. koeln	IV, V	y	1, 2
	S. kaapstad	IV	e, h	1, 7
	S. derby	(I), IV	f, g	
	S. essen	IV	g, m	
	S. budapest	I, IV	g, t	
	S. california	IV	g, m, t	
	S. brandenburg	IV	l, v	e, n, z_{15}
	S. bispebjerg	I, IV	a	e, n, x
	S. abortus equi	IV		e, n, x
	S. arechavaleta	IV, (V)	a	1, 7
	S. abortus ovis	IV	c	1, 6
	S. altendorf	IV	c	1, 7
	S. texas	IV, V	k	e, n, z_{15}
	S. stanleyville	IV, V	z_4, z_{23}	
	S. oahu	IV, V	l, v	1, 2, 3
	S. abortus bovis	(I), IV, XXVII	b	e, n, x
	S. bredeney	I, IV, (XXVII)	l, v	1, 7
	S. schleissheim	IV, XXVII	b, z_{12}	
	S. schwarzengrund	I, IV, XXVII	d	1, 7
C-1	*S. paratyphi C*	VI, VII, (Vi)	c	1, 5
	S. cholerae-suis	VI, VII	(c)	1, 5
	S. typhi-suis	VI, VII	(c)	1, 5
	S. thompson	VI, VII	(k)	1, 5
	S. montevideo	VI, VII	g, m, s	
	S. oranienburg	VI, VII	m, t	
	S. virchow	VI, VII	r	1, 2, 3
	S. oslo	VI, VII	a	e, n, x
	S. amersfoort	VI, VII	d	e, n, x
	S. braenderup	VI, VII	c, h	e, n, z_{15}
	S. potsdam	VI, VII	l, v	e, n, z_{15}
	S. bareilly	VI, VII	y	1, 5
	S. hartford	VI, VII	y	e, n, x
	S. mikawashima	VI, VII	y	e, n, z_{15}
	S. tennessee	VI, VII	z_{29}	
	S. infantis	VI, VII	r	1, 5
	S. concord	VI, VII	l, v	1, 2, 3

(The somatic antigens for the B group species from *S. paratyphi B* through *S. schwarzengrund* are bracketed together with XII.)

* Naturally occurring diagnostic formulae; experimental phase alterations not taken into account.

Legend: ( ) — antigen may be absent; [ ] — incomplete antigen.

THE SALMONELLA TYPES (SPECIES)—*Continued*

Immunologic Types		Antigenic Formulae*		
			Flagellar Antigens	
		Somatic Antigens		
Group	Species		Phase 1	Phase 2
C-1 (Cont.)	S. georgia	VI, VII	b	e, n, z_{15}
	S. papuana	VI_1, VI_2, VII	r	e, n, z_{15}
	S. richmond	VI, VII	y	1, 2, 3
	S. cardiff	VI, VII	k	1, 10
	S. daytona	VI, VII	k	1, 6
	S. mission	VI, VII	d	1, 5
	S. singapore	VI, VII	k	e, n, x
C-2	S. newport	VI, VIII	(e, h)	1, 2, 3
	S. kottbus	VI, VIII	e, h	1, 5
	S. muenchen	VI, VIII	d	1, 2
	S. mexicana	VI, VIII	d	1, 2, 4
	S. pueris	VI, VIII	e, h	1, 2
	S. oregon	VI, VIII	d	1, 2, 3
	S. manhattan	VI, VIII	d	1, 5
	S. litchfield	VI, VIII	l, v	1, 2, 3
	S. morbificans bovis	VI, VIII	r	1, 5
	S. narashino	VI, VIII	a	e, n, x
	S. bonariensis	VI, VIII	i	e, n, x
	S. glostrup	VI, VIII	z_{10}	e, n, z_{15}
	S. duesseldorf	VI, VIII	z_4, z_{24}	
	S. tallahassee	VI, VIII	z_4, z_{32}	
	S. gatuni	VI, VIII	b	e, n, x
	S. hidalgo	VI, VIII	r	e, n, z_{15}
	S. iwo-jima	VI, VIII		1, 5
	S. amherst	(VIII)	l, v	1, 6
	S. virginia	(VIII)	d	
D	S. typhi	IX, (Vi)	d	
	S. enteritidis	(I), IX	g, m	
	S. dublin	I, IX	g, p	
	S. rostock	I, IX	g, p, u	
	S. moscow	IX	g, q	
	S. blegdam	IX	g, m, q	
	S. berta	IX	f, g, t	
	S. pensacola	IX	g, m, t	
	S. claiborne	I, IX	k	1, 5
	S. sendai	(I), IX	a	1, 5
	S. loma-linda	IX	a	e, n, x
	S. durban	IX	a	e, n, z_{15}
	S. onarimon	I, IX ⎫ XII	b	1, 2
	S. eastbourne	(I), IX	e, h	1, 5
	S. panama	I, IX	l, v	1, 5
	S. dar-es-salaam	I, IX	l, w	e, n
	S. goettingen	IX	l, v	e, n, z_{15}
	S. javiana	(I), IX	l, z_{28}	1, 5
	S. gallinarum	IX		
	S. pullorum	IX		
	S. canastel	IX	z_{29}	1, 3, 5
	S. italiana	IX	l, v	1, 11
	S. napoli	(I), IX	l, z_{13}	e, n, x
	S. new york	IX	l, v	1, 5
	S. miami	IX ⎭	a	1, 5

THE SALMONELLA TYPES (SPECIES)—*Continued*

Immunologic Types		Antigenic Formulae*		
			Flagellar Antigens	
		Somatic Antigens		
Group	Species		Phase 1	Phase 2
E-1	S. *london* (L II)	III, X, XXVI	l, v	1, 6
	S. *give*	III, X, XXVI	l, v	1, 7
	S. *uganda*	III, X, XXVI	l, z_{13}	1, 5
	S. *anatum*	III, X, XXVI	e, h	1, 6
	S. *muenster*	III, X, XXVI	e, h	1, 5
	S. *nyborg*	[III, X, XXVI]	e, h	1, 7
	S. *vejle*	III, X, XXVI	e, h	1, 2, 3
	S. *maleagridis*	[III, X, XXVI]	e, h	1, w
	S. *shangani*	III, X, XXVI	d	1, 5
	S. *zanzibar*	[III, X, XXVI]	k	1, 5
	S. *amager*	III, X, XXVI	y	1, 2, 3
	S. *lexington*	[III, X, XXVI]	z_{10}	1, 5
	S. *weltevreden*	III, X, XXVI	r	z_6
	S. *orion*	III, X, XXVI	y	1, 5
	S. *butantan*	III, X, XXVI	b	1, 5
	S. *saipan*	III, X, XXVI	z_6	1, 6
E-2	S. *newington*	III, XV	e, h	1, 6
	S. *selandia*	III, XV	e, h	1, 7
	S. *new brunswick*	III, XV	l, v	1, 7
	S. *illinois*	[III], [XV], XXXIV	z_{10}	1, 5
	S. *cambridge*	III, XV	e, h	1, w
E-3	S. *senftenberg*	I, III, XIX	g, s, t	
	S. *niloese*	I, III, XIX	d	z_6
	S. *simsbury*	I, III, XIX	z_{27}	
	S. *taksony*	I, III, XIX	i	z_6
F	S. *heves*	VI, XIV, XXIV	d	1, 5
	S. *carrau*	VI, XIV, XXIV	y	1, 7
	S. *onderstepoort*	[I], VI, XIV, XXV	e, [h]	1, 5
	S. *florida*	[I], VI, XIV, XXV	d	1, 7
	S. *madelia*	[I], VI, XIV, XXV	y	1, 7
	S. *sundsvall*	(I), VI, XIV, XXV	z	e, n, x
	S. *kentucky*	[VIII], XX	i	z_6
	S. *aberdeen*	XI	i	1, 2, 3
	S. *rubislav*	XI	r	e, n, x
	S. *pretoria*	XI	k	1, 2, 3
	S. *solt*	XI	y	1, 5
	S. *luciana*	XI	a	e, n, z_{15}
	S. *venezia*	XI	i	e, n, x
	S. *senegal*	XI	r	1, 5
	S. *marseille*	XI	a	1, 5
	S. *chandans*	XI	d	e, n, x
	S. *grumpensis*	XIII, XXII	d	1, 7
	S. *poona*	XIII, XXII	z	1, 6
	S. *borbeck*	XIII, XXII	l, v	1, 6
	S. *mississippi*	I, XIII, XXIII	b	1, 5
	S. *wichita*	I, XIII, XXIII	d	
	S. *havana*	I, XIII, XXIII	f, g	
	S. *worthington*	I, XIII, XXIII	l, w	z
	S. *cubana*	I, XIII, XXIII	z_{29}	
	S. *orientalis*	XVI	k	e, n, z_{15}
	S. *hvittingfoss*	XVI	b	e, n, x

THE SALMONELLA TYPES (SPECIES)—*Concluded*

Immunologic Types		Antigenic Formulae*		
		Somatic Antigens	Flagellar Antigens	
Group	Species		Phase 1	Phase 2
F (Cont.)	S. gaminara	XVI	d	1, 7
	S. szentes	XVI	k	1, 2, 3
	S. kirkee	XVII	b	1, 2
	S. cerro	XVIII	z_4, z_{23}, z_{25}	
	S. memphis	XVIII	k	1, 5
	S. minnesota	XXI, XXVI	b	e, n, x
	S. tel aviv	XXVIII	y	e, n, z_{15}
	S. pomona	XXVIII	y	1, 7
	S. hormaeche	XXIX (Vi)	z_{30}, (z_{31})	
	S. ballerup	XXIX, (Vi)	z_{14}	
	S. urbana	XXX	b	e, n, x
	S. adelaide	XXXV	f, g	
	S. monschaui	XXXV	m, t	
	S. inverness	XXXVIII	k	1, 6
	S. champaign	XXXIX	k	1, 5
	S. waycross	XLI	z_4, z_{23}	

one another in several respects. The flagellar antigen is the more unstable and is destroyed by boiling and by exposure to alcohol or weak acid; somatic antigen, on the other hand, is stable to boiling, alcohol and acid. Cultures on phenol agar (0.1 per cent) of bacteria normally containing both H and O antigens are found to contain only O antigens, the formation of flagellar antigen having been suppressed; H antigen reappears immediately on cultivation on nutrient agar. In the agglutination reaction, bacteria lacking flagellar antigen are characteristically clumped in a finely granular precipitate (O agglutination), while bacteria containing flagellar antigen are agglutinated in a coarse, flocculent precipitate (H agglutination).

The H and O agglutination titer of an antiserum may be determined by the use of H and O antigens in the agglutination test. H antigen is commonly prepared by adding an equal volume of formol (0.6 per cent formalin) saline to an eighteen- to twenty-four-hour broth culture. In the preparation of somatic antigen the flagellar component is destroyed by treatment with alcohol; the growth from an eighteen- to twenty-four-hour agar slant culture is emulsified in 1 to 2 ml. of absolute alcohol, heated at 60° C. for one hour, centrifuged and the sediment suspended in 0.5 to 1 ml. saline. It may be used for slide agglutination or appropriately diluted for macroscopic agglutinin titrations.

These two types of antigen are immunologically independent, and the immunization of an animal with a microorganism containing both results in the production of antibodies to both. There is, however, a marked difference in titer, for the O antibody titer is generally much lower than that of the H antibody; in the writer's laboratory antisera having H titers of 1 : 20,000 to 1 : 50,000 have shown O titers of 1 : 2000 or less.

SPECIFIC AND NON-SPECIFIC FLAGELLAR ANTIGENS. The flagellar antigen is, in turn, of dual nature. One kind, designated as *specific flagellar antigen*, is individualistic and contributes in no small part to the immunological identity of a given Salmonella species. The other, termed *non-specific flagellar antigen*, is made up of a limited number of components which are frequently shared by the various Salmonellas and hence contribute to the immunological relationship between the species of these bacteria. The specific flagellar antigens are arbitrarily designated by lower case letters (the choice was unfortunate since the more recently discovered antigens are designated z_1, z_2, z_3, etc.), and the non-specific ones by Arabic numerals.

The antibodies to these antigens behave independently; in the presence of homologous H and O antibodies heated antigen agglutinates in the characteristic fine granular form, while with unheated bacilli coarse flocculation occurs, mixed, within the range of the O antibody titer, with the granular clumps. The antibodies may be selectively absorbed by appropriate antigens, and such absorbed sera are sometimes designated *mono-specific sera*. By the absorption technique using homologous, heterologous or treated antigens, the antigenic components of the bacterial cell may be determined, and through manipulations of this kind, termed *antigenic analysis* (p. 300), White,[4] Kauffmann[7] and others have elucidated the complex antigenic structure and interrelationships of the bacteria of the Salmonella group.

Antigenic Formulae. By identifying these antigenic components it becomes possible to write a formula that describes the immunological character of a given species or strain. Such formulae for antigenic structures are commonly used and are illustrated in the accompanying table.

Classification. While the bacteria given the common generic name Salmonella are obviously closely related to the other members of the enteric group, the interrelationships of the varieties or types of these bacilli to one another is not altogether clear. As indicated above, prior to the emphasis upon immunological character which has been marked since the 1920's, bacteria of this group were separated from one another on the basis of physiological characters. In the more commonly occurring varieties, at least, there is a marked association between biochemical properties and immunological constitution. It would seem neither justified nor desirable to subordinate completely the physiology of these bacteria to their antigenic character.

However this may be, the extent to which antigenic composition should be allowed to determine Salmonella species is open to question. At first it was customary to consider a new immunologic type as a new species, and many of these were given place names. As a consequence a great number of species have been described and continue to be, at the rate of several every year, and at the present time the situation has become absurd. Simplification is obviously essential, but what form it will take is as yet not clear.

Variation. Possibly because the antigenic structure of the Salmonella group is known in considerable detail, two general types of variation may be differentiated. One of these is a type of fluctuating, completely reversible immunological variation known as phase variation, and the other the S–R dissociation known to occur in practically all bacteria.

[7] *Cf.* Kauffmann: Ztschr. f. Hyg. u. Infektionskr., 1937, *120*:177.

Phase Variation. The *H* antigens of certain Salmonella types can be sep-
arated into relatively stable components. This was first demonstrated by An-
drewes[8] and may be shown very simply by plating out such a type and carrying
out slide agglutination of individual colonies with monospecific antiserum.
About half the colonies will be found to contain one kind of antigen and
about half the other. Apparently the individual bacterial cells do not contain
both kinds of *H* antigen but are of two kinds in this respect.[9] This im-
munologic character breeds true to only a limited extent, for if a colony is
picked and subcultured in broth, platings of successive broth cultures will show
a rapid reversion to the 50 : 50 ratio within a very few transfers. This type
of immunological variation is called *phase variation* and the transitory im-
munological types were originally called the *specific phase*, *i.e.*, that charac-
terized by the presence of specific flagellar antigens, and the *non-specific phase*,
i.e., that in which the non-specific antigens occurred. This division is not as
sharp as once thought, however, and now these phases are commonly referred
to as *phase 1* and *phase 2* respectively.

The Salmonella types that exist in two immunological phases are termed
diphasic, while those that exist in only one phase, which may be either phase
1 or phase 2, are called *monophasic*. There is reason to believe that the mono-
phasic types are potentially diphasic, and possibly they are degenerated diphasic
forms, for it has been shown that *S. paratyphi A,* a monophasic type stable
in phase 1, can be induced to form phase 2 antigens by cultivation in anti-
serum specific to the phase 1 antigens. Conversely, diphasic types can be
stabilized in one phase by cultivation in the presence of antiserum to the
antigens of the other phase. By this means Salmonella "species" may be trans-
formed; Edwards, Moran and Bruner,[10] for example, have so transformed S.
simsbury into *S. senftenberg*. Furthermore, "new" species may be created
from monophasic types by suppression of flagellar antigen through cultivation
in the presence of monospecific antiserum, with the appearance of a hitherto
unknown flagellar phase. Such an induced antigen in S. *minnesota* was later
found to occur naturally.[11] Phase variation does not occur, of course, in S.
pullorum which is non-motile and contains no flagellar antigen.

Phase variation is somewhat more complex than this, however, and three
types have been described. The first is the specific-non-specific phase varia-
tion of Andrewes[8] in which a specific antigen ordinarily occurring in phase 1
is linked with the non-specific antigens of phase 2. The second is the so-
called α-β phase variation described by Kauffmann and Mitsui[12] in which an
antigen occurring in phase 1 is linked with the antigens e, n, +, in phase 2.
Five types of α-β phase variation have been reported by Edwards and Bruner.[13]
Lastly, there is a type of phase variation which has not been named in which
the antigens of both phases are commonly found in phase 1.[14]

It is not clear whether this type of immunological variation occurs gen-

[8] Andrewes: Jour. Path. Bact., 1922, 25:505.
[9] *Cf.* the studies of Archer: Jour. Roy. Army Med. Corps, 1941, 77:188.
[10] Edwards, Moran and Bruner: Proc. Soc. Exp. Biol. Med., 1947, 66:230.
[11] Edwards, Moran and Bruner: Proc. Soc. Exp. Biol. Med., 1946, 61:242.
[12] Kauffmann and Mitsui: Ztschr. f. Hyg. u. Infektionskr., 1930, 111:740.
[13] Edwards and Bruner: Jour. Inf. Dis., 1941, 69:220.
[14] Edwards and Bruner: Jour. Hyg., 1938, 38:716.

erally with respect to the somatic antigens. A similar variation, known as *form variation*, does occur, however. Kauffmann[15] has found that of the three components of XII, designated XII_1, XII_2, and XII_3, XII_2 varies in that it is either strongly or weakly developed. Antigen VI is likewise subdivided into VI_1 and VI_2 but similar variation in these latter components has not as yet been reported.

It is to be emphasized that the immunological changes associated with phase variation are, so far as is known, normal, the bacterial strain seemingly existing in a sort of immunological equilibrium. These variations and the complete but temporary loss of flagellar antigen by cultivation on phenol agar noted above apparently bear no relation to the S–R dissociation though, as indicated, they may be induced by a method which will induce dissociation, *i.e.*, cultivation in specific antiserum. There also seems to be some tendency to natural segregation of antigenic components. Edwards[16] has observed such a segregation in the *H* antigens, z_{30}, z_{31}, of *S. hormaechi*; the components appeared to segregate in that for a period of time variants containing only z_{30} occurred and later variants containing only z_{31} were thrown off, both of which were stable and bred true. It is not unlikely that such variation occurs in nature on the one hand, and that the monophasic types represent loss variants, giving rise to a multiplicity of antigen combinations. The phylogenetic implications of the pattern of distribution of antigens within the Salmonella group and of phase variation have been discussed by White,[4] Edwards[3] and Bruner and Kauffmann[15] but cannot be considered here.

Dissociation. S–R dissociation, similar in all respects to that known in other bacteria, occurs in cultures of these bacilli. The dissociation from smooth to rough is manifested as an alteration in colonial morphology and loss of virulence. The change is reflected immunologically as a loss of specificity of the somatic antigens, *i.e.*, the rough forms remain motile. The specificity of these antigens is apparently determined by a polysaccharide haptene, and with the disappearance of the haptene the bacteria acquire a new and common immunologic character in the somatic antigens, while the flagellar antigens remain unchanged. A mucoid or M phase in colonial morphology has been reported by some workers which is said to be associated with the development of a new immunological specificity.

Bacteriological Diagnosis of Salmonella Infection. The differentiation of the paratyphoid fevers from typhoid fever and the determination of the etiology of gastro-enteritis caused by Salmonella is necessarily dependent on the isolation and identification of the causative microorganism.[17] For isolation both enrichment culture and direct plating should be used; enrichment broth and differential selective agar plates should be inoculated simultaneously and, if the latter are negative, fresh plates can be inoculated from the enrichment culture. It is commonly observed that no single agar medium suffices, for with very few bacteria cultures may be isolated on one medium but not the others; at least two, and better three, kinds of differential agar should be used.

Two enrichment media are commonly used. Selenite-F broth contains 0.4

[15] Kauffmann: Jour. Bact., 1941, 41:127.
[16] Edwards: Jour. Bact., 1946, 51:523.
[17] Isolation procedures are discussed in some detail by Littman: War Med., 1943, 4:31.

per cent sodium acid selenite, which is toxic for all gram-negative bacteria but is detoxified sufficiently in the presence of 1 per cent sodium phosphate to permit rapid growth of enteric pathogens while temporarily (eight to twelve hours) inhibiting *Bact. coli*. Tetrathionate broth contains thiosulfate, tetra-thionate and iodide, the tetrathionate being formed by oxidation of the thio-sulfate with iodine. The differential selective agars used contain lactose and an indicator (often neutral red) together with bile or bile salts. Of these the most commonly used are desoxycholate-citrate agar (D–C), Shigella-Sal-monella agar (S–S), and MacConkey agar. The bismuth-sulfite medium of Wilson and Blair and eosine-methylene blue agar (EMB) are more useful for typhoid and dysentery bacilli respectively but are often inoculated also. On the first three the non-lactose-fermenting bacteria form colorless opaque or trans-lucent colonies which are readily differentiated from the red colonies of the lactose-fermenters.

DIFFERENTIAL REACTIONS OF SALMONELLA SPECIES

Species	Xylose	Treha-lose	Arab-inose	Dulcitol	Inositol	H₂S
S. paratyphi A	—	+	+	+*	—	—
S. paratyphi B	+	+	+	+	±	+
Suipestifer group:						
Para C (Hirschfeld)	+	+	+	+	—	+
Kunzendorf (European)	+	—	—	—	—	+
Cholerae-suis (American)	+	—	—	—	—	—
S. typhi-murium (aertrycke)	+	+	+	+	±	+
S. enteritidis	+	+	+	+	±	+

* Usually delayed.

Typical colonies are picked and subcultured in dextrose, lactose and sucrose sugar broths, tryptophane broth, litmus milk and buffered peptone water (for the Voges-Proskauer test), and gram-stained smears are examined. Positive lactose (and/or sucrose) fermentation, positive Voges-Proskauer test, or the formation of indol excludes Salmonella. Litmus milk should become alkaline in four to seven days. Lactose broth cultures should be retained for not less than two weeks to eliminate slow lactose fermentation. Morphologically typical bacilli giving characteristic reactions in these media may be provisionally iden-tified as pathogenic enteric bacilli. Biochemical differentiation may be carried further with additional sugar broths, tests for gelatin liquefaction and hydrogen sulfide formation. This general procedure may be simplified by initial sub-culture in media such as Russell's double sugar agar or Hajna's triple sugar iron agar which allow a tentative separation into coliform, Salmonella, Shigella and *Alcaligenes fecalis* as indicated elsewhere (p. 417). Salmonella is dif-ferentiated from Proteus by failure to hydrolyze urea.

A tentative immunological identification may be made by agglutination with polyvalent antiserum prepared by immunizing with all known Salmonella antigens,[18] but paracolon bacilli which contain Salmonella antigens are ag-

[18] Ewing and Bruner: Jour. Bact., 1947, 53:362.

glutinated as well as Flexner dysentery bacilli of Types I and II because of their immunological relation to O antigens XIII, XXII, and XXIII.

Salmonella Typing. With the definition of Salmonella types as immunological types, complete identification requires the demonstration of at least part of the antigenic structure of the unknown bacterium. This is readily accomplished by slide agglutination with monospecific antisera. If the serological identification includes the rare types, a very considerable number of antisera are required. According to Bornstein,[19] however, more than 95 per cent of the strains encountered in this country can be identified with relatively few antisera. These include six O antisera: II; IV, V; VI, VII; VI, VIII; IX; III, X, XXVI; eleven monospecific H antisera: a, b; i; e; e, h; f, g; m, t; y; g, m, s; l, v; l, 2; and a Vi antiserum. The dilution of the antiserum used in slide agglutination depends upon its titer; in the writer's laboratory 1/100 of the macroscopic titer gives rapid and reliable slide agglutination. Salmonella typing has very considerable epidemiological value and is becoming more and more generally used.

Ecology. The distribution of Salmonella types in man and higher animals has been a matter of considerable interest in recent years with the more general application of serological typing. Aside from S. *paratyphi A*, which appears to be almost exclusively a human pathogen, and S. *paratyphi B*, which is found only occasionally in animals, these bacteria have been regarded as primarily parasites of birds and other animals; some, such as S. *pullorum*, produce a characteristic symptom complex. It has become increasingly clear, however, that many of the Salmonella types are found in man associated with pathological conditions, and it seems probable that transmission from animals, especially fowl, to man and from man to man occurs with some frequency. Edwards and Bruner[20] have reported that of 2520 cultures of Salmonella (exclusive of S. *pullorum* and S. *gallinarum*), 1258 were isolated from fowls, 532 from man, 475 from swine, 90 from rodents, 88 from carnivores, 53 from horses, and 20 from ruminants. The cultures from human sources included 41 types of Salmonella; of those occurring more frequently 132 were S. *paratyphi B*, 60 were S. *typhimurium*, 53 were S. *newport*, 37 were S. *newington*, 28 were S. *cholerae-suis* var. *Kunzendorf*, 27 were S. *panama* and 24 were S. *montevideo*. If these results are in any way representative, as is highly probable, it is apparent that the Salmonella types are widely distributed in man as well as in animals.

Pathogenicity for Man. Two main types of Salmonella infection in man may be distinguished. These are the slow continued fevers of the typhoid type, *i.e.*, the paratyphoid fevers, and the sudden, usually transient, stormy gastro-intestinal disturbances of the food poisoning type. In addition to these a wide variety of clinical diseases is occasionally associated with Salmonella infection, including a generalized invasion with bacteremia, bone infections and other localized infections, infections of the central nervous system, pneumonia and the like. The healthy carrier state in man appears to be considerably more common than has been thought and carrier transmission of infection

19 Bornstein: Jour. Bact., 1942, 44:719.
20 Edwards and Bruner: Jour. Inf. Dis., 1943, 72:58.

undoubtedly occurs. Edwards, Bruner and Moran[21] have reported that, of 2949 cultures isolated from man, 184 or 6.2 per cent were from blood and the remainder from feces. Of the latter group 1597 or 54.1 per cent were from cases of gastro-enteritis, 873 or 29.6 per cent from asymptomatic carriers, 212 or 7.2 per cent from cases of enteric fever, and 83 or 2.8 per cent from miscellaneous infections. Of the Salmonella types only the more important can be considered here.
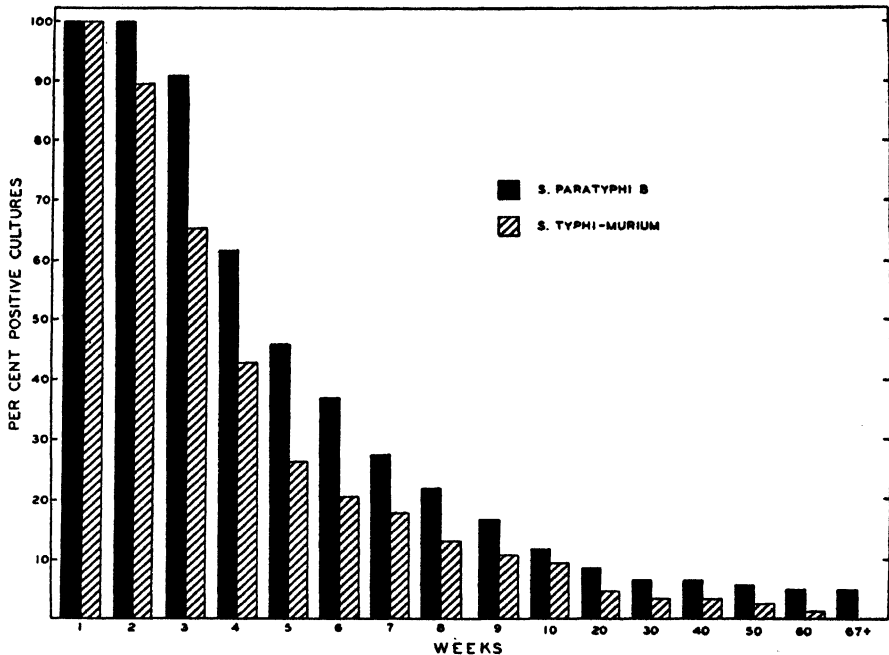
Fig. 72. The persistence of Salmonella infection as indicated by positive fecal cultures by weeks. Data on 203 cases of S. *paratyphi* B infection and 239 cases of S. *typhi-murium* infection by Rubenstein, Feemster and Smith: Amer. Jour. Pub. Health, 1944, 34:841.

The Paratyphoid Fevers. Achard and Bensaude in 1896 isolated from human tissues during convalescence from a typhoid-like disease a bacillus that resembled the typhoid bacillus but differed from it in important particulars. In 1898 Gwyn reported a case, which apparently presented all the clinical symptoms of typhoid fever, in which a bacillus closely related to S. *enteritidis* was isolated from the blood. Since that time a number of investigators in different parts of the world have isolated similar bacteria from the blood of patients suffering from a disease that, so far as clinical symptoms are concerned, is substantially identical with typhoid fever.

Many cases of paratyphoid infection show a tendency to run a relatively mild course and are marked by a sudden onset with chills, but are otherwise very similar to infections with the typhoid bacillus. A certain proportion of

[21] Edwards, Bruner and Moran: Jour. Inf. Dis., 1948, 83:220.

the negative agglutination tests in apparent typhoid fever are probably a result of paratyphoid infection. The only method by which typhoid and para-typhoid fever can be distinguished is isolation and identification of the causal microorganism.

Many scattered cases of paratyphoid fever have been observed, and a number of more or less extensive epidemics have been reported as due to milk and other foods, to contact with human carriers, to sewage-polluted water and similar factors. In general, the mode of dissemination of paratyphoid fever is practically identical with that of typhoid fever (p. 457).

The frequency of the paratyphoid fevers as compared with typhoid fever varies a good deal in different localities, but most hospital records give a ratio of less than 1 : 10. In some regions the proportion of paratyphoid to typhoid may be as high as 1:4 or even more. During the first World War the proportion of paratyphoid to typhoid reached a high point. In the British armies in France during the years 1915–1918, the diagnosed paratyphoid fevers outnumbered the typhoid cases 2: 1. In civilian populations in most countries paratyphoid fevers probably amount to 5 or 10 up to 50 per cent or more of all *fully diagnosed* enteric cases. Very young individuals appear to be the most susceptible to Salmonella infection, and an unduly large portion, perhaps 20 per cent, of cultures are from young children.

Three different species have been commonly recognized as the cause of paratyphoid fever: *S. paratyphi A, S. paratyphi B* and *S. paratyphi C*. It may be noted that a mixed vaccine is commonly used in the prophylactic inoculation against typhoid fever which includes not only typhoid bacilli but *para A* and *para B* bacilli also (p. 461); *para C* is not ordinarily included.

Salmonella Paratyphi A (Bacillus paratyphosus A, Bacterium paratyphosum A, Salmonella paratyphi). This bacterium differs culturally from most of the other Salmonella species in its inability to ferment xylose, and it is, in addition, serologically distinct. Some outbreaks due to this microorganism have been traced to sewage-contaminated water supplies, others to food contaminated through the agency of human carriers. Paratyphoid fever due to *S. paratyphi A* is often very mild, 300 cases occurring in a United States infantry regiment without a single death.

Salmonella Paratyphi B (Bacillus paratyphosus B, Bacterium paratyphosum B, Salmonella schottmülleri), *S. paratyphi B* is readily differentiated from *S. paratyphi A,* but has confusing cultural and serological relations with certain Salmonella strains of the food poisoning types. As in the preceding species, the sources and modes of transmission are similar to those of typhoid fever. The source of the bacteria is generally the human carrier, but it has been reported that dogs[22] have been found responsible for small epidemics and in another instance a cow[23] was responsible for cases of the disease. In the northern United States and in northern Europe infection with this species seems considerably more frequent than with *S. paratyphi A.*

Salmonella Paratyphi C (S. hirschfeldii). *S. paratyphi C* has been found in

---

[22] Caspersen: Norsk Mag. f. Laegevidenskapen., 1937, 98, Forh. Norske Med. Selskab, 138; Ztschr. f. Hyg. u. Infektionskr., 1938, *120:*611; Magnusson: Ztschr. f. Hyg. u. Infektionskr., 1938, *121:*136.
[23] Rosgen and Schultze-Gahmen: Deut. med. Wchnschr., 1939, *65:*1514.

enteric fevers in parts of Asia, Africa and southeastern Europe and has been reported as an important cause of illness and death in British Guiana. Cases of endocarditis have been found to be caused by this and other suipestifer species. Although the type of disease is similar to the other enteric or intestinal forms, little is known about its epidemiology. This species is closely related biologically to the hog cholera or *cholerae-suis* strains described below.

Other species have been occasionally found: *S. barielly*, isolated from cases of mild pyrexia in India; *S. enteritidis* var. *moscow*, isolated from cases of paratyphoid fever in Russia and described by Russian bacteriologists as "Paratyphus N_2"; *S. sendai*, isolated from cases of paratyphoid fever in Japan; and *S. eastbourne*, from a paratyphoid case of Eastbourne, England. In this country *S. typhi-murium, S. saint paul, S. oranienburg, S. hartford, S. sendai, S. panama* and others have been found associated with enteric fevers.

Paratyphoid Gastro-enteritis. The symptoms of this type of disease are quite different from those in paratyphoid fever and comprise a more or less violent gastro-intestinal disturbance with vomiting, diarrhea, a slight rise in temperature and usually a rapid recovery. The attack may rarely pass into a septicemic infection. The descriptions of indigenous cholera or "cholera nostras" in earlier medical writings suggest this form of illness. Outbreaks of gastro-enteritis have been usually reported in connection with the consumption of particular articles of food and are commonly referred to as "food poisoning." Food-borne infection of this type has been discussed elsewhere (p. 273) and need not be considered further here. Several species of bacteria are known to be concerned.

Salmonella Typhi-murium (Salmonella aertrycke, Bacterium aertrycke, Bacterium typhi-murium). This bacterium, most commonly isolated in food poisoning outbreaks in the United States[24] and in Great Britain,[25] closely resembles *S. paratyphi B* in its cultural characteristics but can be distinguished by its ability to produce acid in tartrate medium. Before differential tests were satisfactorily worked out, *S. paratyphi B* and *S. typhi-murium* were commonly confused and both termed "para B." *S. typhi-murium* is commonly found in a variety of infections in laboratory and domestic animals and in birds; and the "*B. pestis caviae*" of some writers and Nocard's "*B. psittacosis*" are, in fact, *S. typhi-murium*. Most of the laboratory stock cultures labeled "Danysz virus" or "bacillus of mouse typhoid" are of the typhi-murium type, but some are *S. enteritidis.*

Salmonella Enteritidis (Bacterium enteritidis). Although found frequently in food-poisoning outbreaks, this bacterium is less common than *S. typhi-murium*. It closely resembles *S. typhi-murium* culturally, but is said by some to differ in that it does not ferment inositol while *typhi-murium* does. The inositol fermentation is, however, frequently not clear-cut; a lowering of pH may be noted but sometimes not to a sufficient degree to warrant calling the fermentation positive. In the writer's experience, the inositol fermentation is variable in both species. The two may be separated by serological means.

Salmonella Cholerae-Suis (Bacterium suipestifer, Bacterium cholerae-suis, the Hog Cholera Bacillus, American Suipestifer). This Salmonella is a mem-

[24] Jordan: Jour. Prev. Med., 1929, 3:279.
[25] Savage and White: Med. Res. Council Spec. Rept. Ser. No. 92, 1925.

ber of a group of closely related bacteria, called the *suipestifer group,* which also contains S. *paratyphi* C or Eastern type, as noted above, S. *cholerae-suis* var. *Kunzendorf* or European type, and the Glässer-Voldagsen type, which is comprised of two species, S. *typhi-suis* and S. *typhi-suis* var. *Voldagsen.* These species may be differentiated from one another by a combination of cultural and serological methods. S. *cholerae-suis* formerly predominated in the United States, but now the Kunzendorf variety is found more often. This species has been implicated in outbreaks of paratyphoid gastro-enteritis, although to a much lesser extent than either S. *typhi-murium* or S. *enteritidis.* S. *typhi-suis* appears to be purely an animal (pig) pathogen, but several cases of human infection with the Kunzendorf variety have occurred in the United States. According to Eschweiler, Wahlin and Snow[26] eighty-six cases of human infection with bacteremia have been reported in this country.

Other Salmonella species are less commonly implicated in human infections. S. *thompson* and S. *newport,* both related to the suipestifer group immunologically, have been observed a few times and other species but once; in fact, a number of the new Salmonella species described in recent years have been isolated from food implicated in outbreaks of enteritis.

The distinction between the "food-poisoning" strains of paratyphoid bacilli and the strains that cause slow typhoid-like fever does not seem to be a sharp one. In rare instances acute gastro-enteritis has been traced to S. *paratyphi B,* and, while illness caused by the food-poisoning bacilli is ordinarily followed by prompt recovery, fatal cases of generalized infection with bacteremia sometimes occur. In general, however, S. *paratyphi A,* S. *paratyphi B* and S. *paratyphi C* are found in the continued fevers; S. *typhi-murium,* S. *enteritidis* and, more rarely, S. *cholerae-suis* in acute gastro-enteritis. Salmonellae of the suipestifer group appear to be more invasive when infecting man than most of the other species, and consequently are more often found in bacteremia and other kinds of tissue infection.

Pathogenicity for Lower Animals. Salmonella infection of rodents is quite common; S. *typhi-murium* and S. *enteritidis* cause infections of rats and mice, and these animals may become healthy carriers of the bacilli, a point of importance in connection with the epidemiology of food-poisoning outbreaks. S. *typhi-murium* infection is by far the most common in the United States, and S. *enteritidis* less so than generally supposed. Preparations of "rat virus" or "Ratin" consist of these bacteria and are supposed to initiate an epidemic of disease in the rat population and hence destroy it. The use of such preparations is to be condemned, for not all the rats are killed and many of the survivors become healthy carriers; there is, in addition, the danger involved in leaving preparations of virulent bacilli about the home.

Salmonella infection of the horse is also quite common. Infectious abortion of mares is caused by a specific microorganism, S. *abortus equi,* which has not been found in other animals. Man is only rarely infected. S. *typhi-murium* has also occasionally been reported in horses. Abortion in sheep has been attributed to a member of the Salmonella group, S. *abortus ovis.* Salmonella is occasionally observed in a variety of other animals.

Birds are quite commonly infected with members of the Salmonella group.

26 Eschweiler, Wahlin and Snow: Ann. Int. Med., 1944, 20:275.

Epidemics due to S. *typhi-murium* sometimes cause great destruction among canaries and other songbirds. Two barnyard diseases of great economic importance are due to specific Salmonella types: the bacillary white diarrhea of chicks caused by S. *pullorum;* and fowl typhoid caused by S. *gallinarum* (or S. *sanguinarium*). S. *pullorum* may survive in the ovaries of the fowls that recover from infection; diseased chicks may develop from the infected ova and communicate the disease to initially healthy members of the flock. Rare cases of human infection with S. *pullorum* have been reported and it has been associated with epidemic food-borne gastro-enteritis in some instances.

THE ENTERIC BACILLI: THE TYPHOID BACILLUS

The infectious nature of typhoid fever was apparent in 1856 to William Budd, who, on the basis of epidemiological evidence, suggested that the disease was transmitted by sewage-contaminated water and that the source of the infectious material was human feces. The typhoid bacillus, however, was discovered in 1880 by Eberth in the mesenteric glands and the spleen of persons dying from typhoid fever. In 1884 Gaffky succeeded in growing Eberth's bacillus on culture media. Acceptance of this microorganism as the etiologic agent of typhoid fever, however, was delayed because typhoid fever could not be reproduced in experimental animals. The immunological aspects of typhoid fever provided strong ancillary evidence, and, in the course of time, Koch's third postulate has been fulfilled by infections in man arising from laboratory accidents.

Morphology and Staining. The typhoid bacillus closely resembles the other enteric bacteria and exhibits no distinctive morphological characters. The microorganism is a short, plump rod, ranging, as a rule, from 1 to 3.5 μ in length and from 0.5 to 0.8 μ in breadth. In smears from agar cultures the shorter forms predominate, while longer bacilli are generally observed in liquid media. It may be noted that the typhoid bacilli present in the urine of urinary carriers are frequently in the form of long filaments. This bacterium is actively motile by peritrichous flagella and generally possesses a greater number of flagella (12 to 14) than the colon bacillus (6 to 10). Spores are not formed, and the cell inclusions taken by Gaffky and others to be spores were probably either vacuoles or metachromatic granules. Capsules are not formed.

Upon agar and gelatin media the colonies of the typhoid bacillus closely resemble those of the colon bacillus and are equally variable in appearance. The "maple-leaf" appearance, arising from the irregularly notched margins and often termed typical, is by no means always seen, and the colonies are frequently round, smooth, bluish white, translucent and slightly raised. The true typhoid bacillus is never pigmented; although there have been a number of reports[1] of the isolation of yellow pigment-forming varieties, these are not true typhoid bacilli.[2] Typical cultures of the typhoid bacillus grow upon the surface of acid potato, but the growth is thin, moist and colorless, and forms the so-called "invisible film" which is strikingly unlike the profuse brownish growth of the typical colon bacillus. On pieces of potato with an alkaline reac-

[1] Grossmann: Centralbl. f. Bakt., I, Orig., 1933, *129*:508; Castro: Deut. med. Wchn-schr., 1934, *60*:1014; Dresel and Herbert: Arch. Hyg. u. Bakt., 1938, *120*:286; Rotenburg: Ztschr. Mikrobiol. Epidemiol. Immunitätsforsch. (U. S. S. R.), 1939, No. 5, 61.

[2] Cruickshank: Jour. Hyg., 1935, *35*:354.

tion, the growth is more like that of the colon bacillus. No great value as yet attaches to the character of growth on potato, for there is wide variation in both the reaction of potatoes and the behavior of different strains of bacilli.

The typhoid bacillus stains easily with the ordinary aniline dyes and is readily decolorized by the Gram method.

Physiology. *Salmonella typhi* is not nutritionally fastidious and grows readily upon the usual nutrient (beef extract) agar and gelatin. These bacteria may be grown on simple synthetic solutions containing glucose and an ammonium salt; some strains appear to require the addition of tryptophane to these solutions,[3] but it is probable that this amino acid functions as a growth stimulant rather than an essential food substance.[4] The typhoid bacillus is a facultative anaerobe, growing almost as luxuriantly under anaerobic conditions
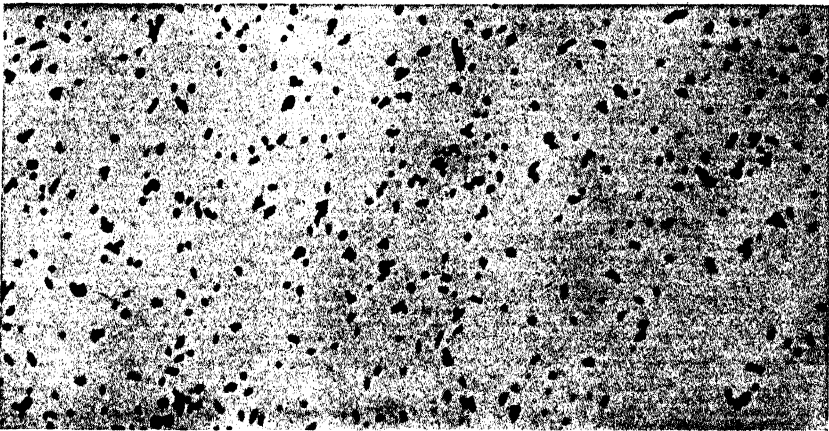
Fig. 73. *Salmonella typhi.* Smear from a pure culture, Sommersby strain. Note the variation in size from coccoid to bacillary forms. Fuchsin; $\times$ 1050.

as under aerobic conditions. Some growth is apparent at 4° C. and there is no growth beyond 46° C., while the optimum temperature is 37° C. Like the other intestinal forms, the typhoid bacillus grows over a relatively wide pH range, 5.0 to 8.6., with an optimum at pH 6.8 to 7.0.

Gelatin is not liquefied and indol is not formed, but nitrates are reduced to nitrites. Hydrogen sulfide is produced. As compared to many of the other enteric forms, the typhoid bacillus is a relatively weak fermenter and it resembles *Bact. coli anaerogenes* in that acid and no gas is produced; this last characteristic is of practical value in the glucose fermentation in the differentiation of the typhoid bacillus and Salmonella species. The acids produced in the fermentation of glucose are lactic acid for the most part, together with small amounts of ethyl alcohol, formic and acetic acids and, sometimes, succinic acid. Acetylmethylcarbinol is not formed. Although no gas may be observed in the fermentation tube, precise measurement has shown that carbon dioxide is evolved under aerobic conditions, one mol of carbon dioxide being given off

[3] Fildes, Gladstone and Knight: Brit. Jour. Exp. Path., 1933, *14*:189.
[4] Burrows: Jour. Inf. Dis., 1939, *64*:145; *ibid.*, 1939, *65*:134.

for every mol of oxygen taken up.[5] Other cultural reactions of the typhoid bacillus are summarized elsewhere (p. 416), but, in general, carbohydrate fermentations, with the exception of the failure to ferment lactose, are not of great differential value.

   The susceptibility of the typhoid bacillus to deleterious influences is much the same as that of the other enteric bacteria. It is killed by exposure to 55° to 60° C. for thirty minutes and by the usual bactericidal chemicals in a somewhat shorter time than certain more resistant forms such as staphylococci. It persists, rather than multiplies, in nature for a variable length of time. Some bacilli remain viable in ground water for possibly two or three weeks, but in fecal matter in privy vaults and elsewhere they may persist for one to two months.

Fig. 74. Colonies of typhoid bacillus on nutrient agar. Note the characteristic "maple-leaf" irregular margin and slightly roughened glistening surface. × 6.

   Toxins. S. *typhi*, like the other enteric bacteria, does not form a soluble toxin, but its cell substance is toxic to the experimental animal upon parenteral inoculation. A polysaccharide-lipid complex which is reported to represent the somatic antigen and endotoxin has been isolated from typhoid bacilli by Boivin and Mesrobeanu[6] by trichloracetic acid extraction (p. 284). A similar substance, but differing in that it contains protein, has been isolated by Morgan and Partridge[7] by extraction with diethylene glycol and purified by precipitation from aqueous solution with acetone. The contained polysaccharide is immunologically specific, reacting with O antisera, and is probably the same as that studied by Freeman.[8] An alcohol-soluble substance having toxic properties has been studied by White[9] and named by him "Q" substance. Similar

   [5] As measured in Warburg respirometers in the writer's laboratory. Carbon dioxide is also given off by aerated broth cultures.
   [6] The work on the typhoid bacillus is discussed by Boivin and Mesrobeanu: Rev. d'Immunologie, 1938, 4:197.
   [7] Morgan and Partridge: Brit. Jour. Exp. Path., 1942, 23:151.
   [8] Freeman: Biochem. Jour., 1942, 36:340.
   [9] White: Jour. Path. Bact., 1932, 35:77; Dennis: Proc. Soc. Exp. Biol. Med., 1939, 42:89.

substances have been found in quite a number of the enteric bacilli and their relationship to the lipid complexes is not clear. Morgan[10] has studied the pathologic changes produced by the endotoxin in some detail. Intradermal inoculation results in a local edema and erythema followed by necrosis, and intravenous or intracardial injection results in congestion, hemorrhagic extravasation and necrosis in various organs. The liver and bone marrow pathology is very similar to that observed in fatal human cases of typhoid fever. The vascular epithelium is injured also and thrombosis, common in severe human infections, is produced.

Classification and Antigenic Structure. The typhoid bacillus has been given a variety of names, including *Bacillus typhi, Bacillus typhosus* and *Bacterium typhosum.* Although closely related to the other enteric bacilli, it was put into the genus Eberthella as the type species, first under the name of *Eberthella typhi* and later as *Eberthella typhosa* in the earlier Bergey classifications. The antigenic structure of the typhoid bacillus is given by the formula IX, XII, (Vi) : d : and its close immunological relation to the Salmonellas is obvious. As a consequence of this relationship, many workers, the English in particular, allocate the typhoid bacillus to this genus and term it *Salmonella typhi.* The current Bergey (1948) classification has accepted this view and now formally classifies the typhoid bacillus as *Salmonella typhosa.* Since the name *Salmonella typhi* has become established to a certain extent, it would seem desirable to retain it.

Vi Antigen.[11] In addition to the usual somatic and flagellar antigens, the majority of strains of typhoid bacilli isolated from human infections, some of which are inagglutinable by O antiserum, were found by Felix and Pitt[12] to contain an additional antigen. This was designated the Vi or "virulence" antigen since they believed it was associated with the virulence of the bacteria. This antigen appears to be similar to the somatic antigens, has in fact been reported[13] to be extracted with trichloracetic acid, but differs in that it is heat labile in the presence of water. It is, however, relatively heat-stable in absolute alcohol, acetone or glycerin.[14] According to Pijper[15] the type of agglutination produced by the antibody to this antigen differs from the normal H and O agglutination in that a sort of "paresis" is produced in the flagella, resulting in erratic movements and chance contacts between the cells. Though there appears to be no mutual attraction, the bacilli seem to be sticky and, when relatively large areas come in contact, they adhere to form clumps in which the bacilli tend to be arranged side by side.

Practically all strains of typhoid bacilli isolated from cases of the disease contain Vi antigen, though exception has been noted, and more than 95 per cent of strains isolated from carriers contain it. The antigen tends to be lost as the strain is carried on laboratory media and it eventually disappears. A series of steps is apparent in this degradation. The strain is at first inagglutinable by O antiserum and the first step is the acquisition of O agglutinability, often after

---

[10] Morgan: Amer. Jour. Path., 1943, *19*:135.
[11] See the review by Almon: Bact. Rev., 1943, *7*:43.
[12] Felix and Pitt: Lancet, 1934, *ii*:186.
[13] Boivin and Mesrobeanu: Compt. Rend. Soc. Biol., 1938, *128*:5.
[14] Peluffo: Proc. Soc. Exp. Biol. Med., 1941, *48*:340.
[15] Pijper: Jour. Path. Bact., 1941, *53*:431.

only one or two transfers. Next the strain loses the ability to stimulate the formation of Vi antibody, then agglutinability with monospecific Vi antiserum disappears, and finally ability to absorb Vi agglutinins from antiserum is lost. Kauffmann[16] has suggested a terminology for these changes which has been generally adopted. A strain inagglutinable in O antiserum is termed a *V strain*, when it agglutinates in O antiserum but retains other Vi characteristics it is a *V-W strain*, and when a strain gives no evidence of the presence of Vi antigen it is designated a *W strain*.

The association of Vi antigen and virulence is not clear-cut, and some workers have expressed doubts of its validity. Virulence is difficult to measure, particularly since typhoid fever is not reproduced in laboratory animals. It may be noted that the same antigen has been found in S. *paratyphi C,* and its presence in these bacteria is not correlated with their virulence (in terms of minimum lethal dose) for mice, their natural host. Nevertheless, Vi antibody has powerful protective properties as assayed by the mouse protection test and appears to be somewhat more efficient in this respect, though qualitatively no different, than O antibody. Antibody to Vi antigen from bacteria other than the typhoid bacillus, such as S. *ballerup,* protects mice against typhoid bacilli as well as antibody to the homologous antigen.

Bacteriophage Typing. It was shown by Craigie and Yen[17] that a number of types of Vi-containing typhoid bacilli may be differentiated on the basis of susceptibility to the lytic action of races of bacteriophage. These types and subtypes are designated by letters, A, B_1, B_2, C, etc., through T to a total of 24 types. There is some cross reaction between subtypes, but very little between types. These phage types are apparently stable. Craigie and Felix[18] have suggested a standardized procedure for phage typing and this or some other should be adopted to assure uniformity in results. Typing is readily accomplished by inoculating a series of areas on an agar plate and, after the inoculated areas have dried, each is inoculated separately with the type phages diluted to act selectively. After incubation an area of lysis is produced by the phage type to which the unknown strain belongs. Phage typing has been of considerable value in epidemiological studies.

Variation. The dissociation of the typhoid bacillus into the usual smooth and rough colonial types is well known. The round, domed, bluish white colonies observed in blood cultures are the typical smooth form, while the rough colonies are flatter, with a roughened surface and irregular edges, and are more opaque. The rough forms are not necessarily non-motile, and in this respect four types may be distinguished: the smooth motile, the smooth non-motile, the rough motile and the rough non-motile. The S-R transformation is, as in the case of the Salmonellas, associated with a change in the immunological specificity of the somatic or O antigens. The Vi antigen is apparently independent and may be present either with or without O antigen. A series of antigenic combinations is, then, possible, for each of the four above types may or may not contain Vi antigen, and H antigen, O antigen and Vi antigen may be present separately or in any combination.

[16] Kauffmann: Ztschr. f. Hyg. u. Infektionskr., 1935, *116*:617.
[17] Craigie and Yen: Canadian Pub. Health Jour., 1938, *29*:448, 484.
[18] Craigie and Felix: Lancet, 1947, *i*:823.

The presence or absence of Vi antigen is not associated with the S-R transformation, and the gradual disappearance of this antigen on continued cultivation on laboratory media cannot be regarded as a dissociative change.

The S-R transformation may take place in the body, and it is a common experience to find that typhoid bacilli isolated from carriers are spontaneously agglutinable, avirulent, typically rough forms. The relation of this transformation to the continued presence of the bacilli in the healthy carrier is not clear, though possibly associated with the S-R transformation that may be brought about *in vitro* by cultivation in the presence of immune serum.

Pathogenicity for Man. Typhoid fever (enteric fever; Ger., *Abdominaltyphus* or *typhus*; Fr., *la fièvre typhoïde*) was for long one of the most widespread and important of all bacterial diseases. In the United States in 1900, there were 35,379 reported deaths from this disease, undoubtedly a low figure, and probably some 350,000 cases of typhoid fever in a population of 76,000,000—in the course of a decade perhaps one person in every 20 to 25 contracted the disease. The prevalence of typhoid fever has greatly diminished in recent years, and a large part of this decrease has taken place in the large cities. The total deaths from typhoid fever in 93 cities in the United States with an aggregate population of 38 million were 385 in 1935, 259 in 1939, 95 in 1942, 85 in 1943, 73 in 1944, and 87 in 1945. In 1945 56 of the cities had no deaths, 31 had less than 1 per 100,000 and only 6 a rate of over 1 per 100,000. In the case of 78 cities for which data are available, the rate has fallen from 20.5 in 1910 to 0.2 in 1945.[19] In the country as a whole, 4425 cases and 472 deaths were reported in 44 states in 1945, rates of 3.8 and 0.4 per 100,000 respectively. Typhoid still persists, however, and epidemics occur from time to time, particularly in the smaller towns and rural areas. Much the same situation prevails in other countries, such as Great Britain.

The common symptoms of typhoid fever include frontal headache, lack of appetite, nosebleed, the development of rose spots on the abdomen, muscular weakness and diarrhea. Sometimes considered primarily an intestinal infection, the disease is, in fact, a general invasion of the body, particularly of the lymphatic system. The intestine is often regarded as the main portal of entry of the bacilli into the body; the lymphatic tissues in the intestinal wall are first invaded and the bacilli spread through the lymphatic system. After considerable multiplication has occurred (incubation period), the bacilli overflow into the blood and bacteriolysis takes place; the endotoxins which are liberated as a result of the destruction of the bacilli produce the symptoms of typhoid fever. Other evidence suggests that the body tissues may be invaded through the tonsils and gastric mucosa, but in any case the end result is the same and typhoid fever is a general and not a localized infection.

The typhoid bacillus appears in the blood stream early in the disease, *i.e.*, after the onset of symptoms, and may be cultured within the first ten days in the majority of cases, either from a blood sample or from the clot of samples sent in to a laboratory for agglutination tests. The presence of ·typhoid bacilli in the blood, however, does not constitute a septicemia; in

[19] Jour. Amer. Med. Assn., 1946, *131*:817.

fact, probably little or no multiplication takes place. The bacilli are also present in the bone marrow early in the disease, and some have urged culture by sternal puncture to facilitate diagnosis. During and after the second week typhoid bacilli may be found with increasing frequency in the feces, and the proportion of positive blood cultures drops off. The bacilli are also excreted in the urine in perhaps 25 per cent of the cases. They may often be found early in the disease in the rose spots, not in the blood but in the lymphatic spaces.

On autopsy, the intestinal walls are usually found to be extensively ulcerated, Peyer's patches and the solitary glands of the intestine being par-
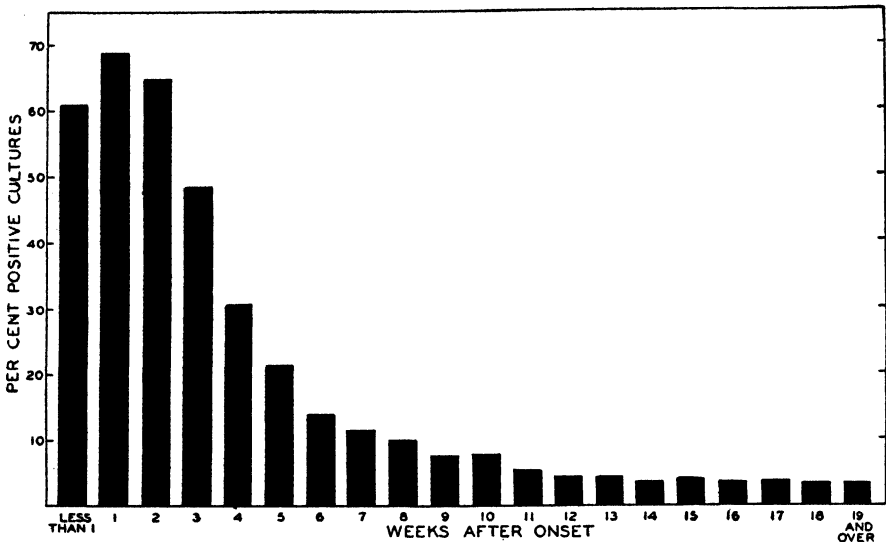
Fig. 75. The persistence of typhoid bacillus infection as indicated by percentage of positive fecal cultures by weeks after onset. Data from 374 cases in New York State exclusive of New York City by Ames and Robins: Amer. Jour. Pub. Health., 1943, 33:221.

ticularly involved and containing typhoid bacilli. Perforation of the intestinal wall as a consequence of ulceration is a not uncommon occurrence. The spleen is enlarged and congested and usually contains large numbers of typhoid bacilli. In both the spleen and the liver the bacilli seen in stained sections occur in groups or masses rather sharply focalized; scattered individuals are not often found.

A variety of complications may occur. Laryngeal ulcer is occasionally observed. The gallbladder is not infrequently infected and cystitis sometimes occurs. Suppurative and inflammatory processes may appear in other parts of the body. The osseous system seems especially open to attack, and affections of the periosteum, the bone marrow and the joints have been traced to infection with *S. typhi*. Osteomyelitis may develop as long as six or seven years after recovery from typhoid fever, indicating that the typhoid bacillus can remain in contact with human tissues for years without losing its virulence. Other parts of the body are more rarely invaded during typhoid fever,

but almost any organ can be attacked occasionally. The presence of *S. typhi* has been reported in a brain abscess, and the cerebral and meningeal symptoms occurring in many cases of typhoid fever are directly connected with the localization of the bacilli in the meninges—bacilli have been found in spinal fluid obtained by lumbar puncture.

Secondary or mixed infections, especially with the pyogenic cocci and the pneumococcus, are not at all uncommon, and sometimes result in serious complications. Mixed infections with the tubercle bacillus and the anthrax bacillus have also been observed.

Carriers. About one-third of the individuals having typhoid fever discharge bacilli for a period of three weeks after the onset of illness and about 10 per cent for eight to ten weeks; these are known as convalescent carriers. A certain proportion continue to discharge typhoid bacilli for six months or more, and in many cases over a period of several years or throughout the whole of a long life.

The development of the carrier condition is probably dependent upon the invasion of the gallbladder in the case of the fecal carriers and of the urinary bladder in the case of the urinary carriers. Fecal carriers are more common than urinary carriers, and combined fecal and urinary carriers are relatively uncommon. It is not known why women are more commonly carriers than men. In the series studied by Ames and Robins[20] 2.1 per cent of the males became chronic carriers as compared with 3.8 per cent of the females. Age is a factor also; according to the same workers the percentage of cases becoming carriers was 0.3 in the 0–9 and 10–19 age groups, but as high as 10.1 in the 50–59 age group. The usual estimates for all age groups vary from 0.5 to 11.6 per cent. Typhoid bacilli need not be excreted continuously; in fact, their intermittent appearance is very common, and weeks may elapse with negative cultures before the bacilli reappear. The necessity for repeated examinations is, of course, obvious. A majority of carriers give the Widal reaction, and in most cases the opsonic index is abnormally high. Antibody to Vi antigen is found in the great majority of carriers, but is only transitory if present at all in inoculated persons, and the use of the Vi agglutination test for the detection of carriers has given encouraging results.

Attempts to cure typhoid carriers by non-surgical means, such as chemotherapy, vaccine therapy or bacteriophage, have not been generally successful, and such procedures are not generally advocated at the present time.[21] Removal of the gallbladder under suitable conditions is often effective in the case of fecal carriers; possibly three-fourths or more are cured.

The proportion of typhoid carriers in the general population is not known, owing to obvious practical and technical difficulties. It is probably quite different in different localities and doubtless depends largely upon the prevalence of current and past typhoid infection. By a modified life table procedure Ames and Robins[20] estimated a carrier rate of 42 per 100,000 in New York State. On the basis of reported deaths and assuming a case fatality rate of 10 per cent and a chronic carrier incidence of 2 per cent in

[20] Ames and Robins: Amer. Jour. Pub. Health, 1943, 33:221.
[21] See the discussion by Cutting and Robson: Jour. Amer. Med. Assn., 1942, *118*:1447; Feemster and Smith: Amer. Jour. Pub. Health, 1945, 35:368.

survivors, Anderson, Hamblen and Smith[22] estimated a carrier rate of 48 per 100,000 in Massachusetts, and Grey[23] similarly estimated a rate of 288 in Mississippi. The number of carriers throughout the United States is probably decreasing since they are no longer produced in such quantities as when typhoid fever was prevalent. By extrapolation Ames and Robins[20] calculated that the 2500 carriers in New York State in 1940 would be reduced to 200 by 1980.

Bacteriological Diagnosis of Typhoid Bacillus Infection. The isolation and identification of the typhoid bacillus is essential in the detection of the carrier state and is necessary to establish a diagnosis of typhoid fever. The isolation of the bacillus from blood or urine specimens is ordinarily a relatively simple matter since the fresh specimen is not heavily con-
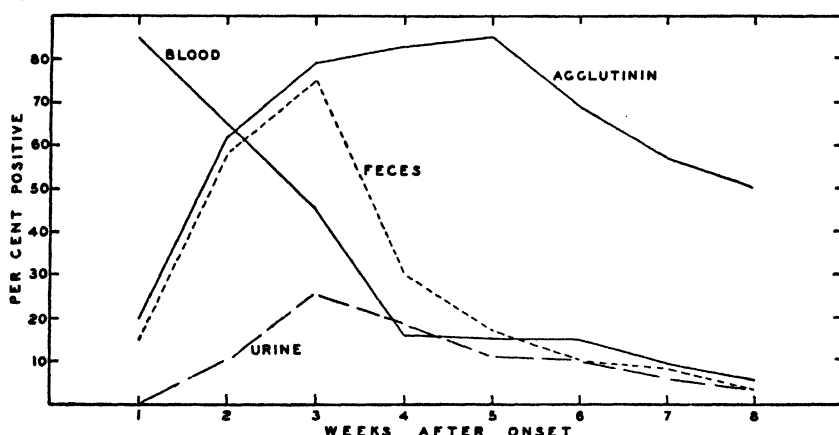
The approximate incidence of positive culture of blood, feces and urine, and agglutinin response in typhoid fever.

taminated. Fecal specimens, however, contain very large numbers of *Bact. coli* and other bacteria and selective as well as differential media are required.

The procedure is essentially the same as that used for the isolation of other Salmonella, and includes enrichment culture in Selenite-F broth and direct plating on S-S and D-C agars and on MacConkey agar. Blood is usually cultured in bile broth or brilliant green bile broth, though cultures in nutrient broth are often satisfactory. The bismuth-sulfite agar of Wilson and Blair is particularly useful for the isolation of the typhoid bacillus for the colonies on this medium are black and distinctive; however, S. paratyphi B and S. enteritidis also grow as black colonies. The medium is strongly inhibitory and must be inoculated very heavily; consequently, colonies may not be pure cultures. Growth on this medium is often not a satisfactory agglutinating antigen. The medium is difficult to prepare in uniform quality but because of its utility is very widely used.

The typhoid bacillus is identified by biochemical reactions and specific

[22] Anderson, Hamblen and Smith: Amer. Jour. Pub. Health, 1936, 26:396.
[23] Grey: Amer. Jour. Pub. Health, 1938, 28:415.

agglutination and must be differentiated from 'other enteric bacilli which cause clinically similar disease.

Epidemiology. The typhoid bacillus is a strict parasite found only in man. Outside the human body multiplication, if it occurs at all, is insignificant, and for practical purposes may be neglected as a factor in the dissemination of the disease. As indicated above, the typhoid bacillus leaves the body in the feces or, less commonly, the urine, and enters the body of a new host via the alimentary tract. The epidemiology of typhoid fever, then, is predicated upon the connection between the intestinal tract of the infected person and the mouth of the susceptible, and the factors that determine the spread of this disease are, essentially, those arising as a consequence of the interrelationships of the individuals or groups of individuals comprising the host population. The extent of the spread of typhoid is, of course, dependent upon the nature of the connecting links between individuals, and two epidemiological types of the disease may be distinguished, the one epidemic typhoid, and the other endemic, or residual, typhoid.

Epidemic Typhoid Fever. Extensive outbreaks of typhoid fever necessarily involve a connecting link that is common to a great many people, and by far the most important vectors of this kind are water and milk. As pointed out elsewhere (Chapter 10), water-borne typhoid fever, formerly all too common but by now relatively rare in the larger communities, arises as a consequence of the contamination of a water supply with infectious fecal material, either as such or in the form of sewage. Water-borne epidemics of typhoid fever occur in the absence of chlorination, filtration and other purification procedures and may, of course, be readily prevented. These epidemics tend to occur in the cold months of the year, particularly in the winter and early spring, and the incidence of the disease is unaffected by age, sex or economic status.

Milk-borne typhoid fever, at the beginning of the twentieth century second only to water-borne typhoid in extent and importance, follows the route of the milkman and, as might be expected, tends to occur in the lower age groups and in families of higher economic status. The general introduction of the process of pasteurization has practically eliminated milk-borne typhoid fever from the larger urban centers, but epidemics continue to occur from time to time in various parts of the country.

Food-borne typhoid fever may take on epidemic proportions in certain instances. Oysters and other shellfish have come into bad repute in this respect in recent years, for a number of typhoid epidemics in Great Britain and the United States have been found to be due to the eating of oysters grown near sewer outfalls or placed to "fatten" in the polluted waters of estuaries or creeks. Watercress, lettuce, radishes or any vegetables or fruits which are liable to come in contact with contaminated water or are sprayed with human excrement may give rise to small-scale epidemic typhoid fever.

Endemic Typhoid Fever. Although epidemic typhoid fever is largely eliminated in a given community through adequate sanitary control of water and milk supplies and such food supplies as are susceptible to the application of effective control measures, the disease remains in an endemic form which is manifested as occasional cases or small groups of cases which

appear from time to time. The seasonal incidence is quite different from that of water-borne typhoid; the marked increase in incidence in late summer and early fall is not explained (Fig. 76). The source of infection is, of course, the case, frank or ambulatory, or the healthy carrier. Instances of direct, contact infection are unquestionably more common than is generally recognized, and the dissemination of typhoid bacilli from the infected individual to his immediate associates is undoubtedly responsible for the majority of cases of residual typhoid. Carriers are, of course, of particular importance in this connection in that they constitute semipermanent foci
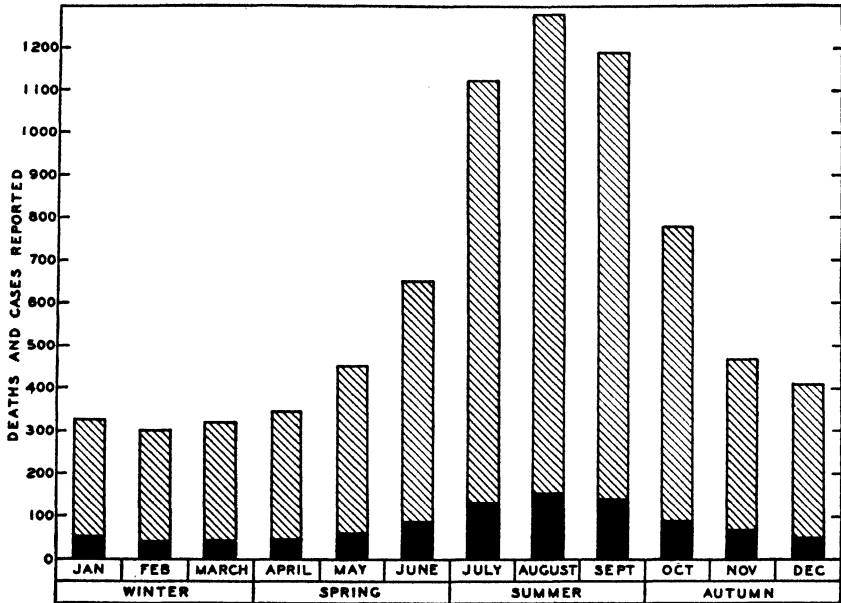
Fig. 76. The seasonal incidence of typhoid and paratyphoid fever. Averages of reported cases by months for the years 1939 to 1945 inclusive. Data from Supplements to Public Health Reports.

of infection and, when employed as food handlers, may be the cause of small epidemics. The most notorious instance of this kind was that of Mary Mallon, "Typhoid Mary," who was unknowingly the cause of some twenty-six cases of typhoid fever in seven different families. Under special circumstances, such as prevail among troops, contact infection may assume epidemic proportions.

The reduction in the prevalence of typhoid fever in the last few decades (Fig. 77) is attributable almost entirely to elimination of the great water-borne and milk-borne epidemics. As noted above, epidemics still occur, and their control is a matter of putting into practice existing knowledge. Residual typhoid, however, is much more difficult to control; the detection and supervison of all carriers, or even the elimination of carriers as food handlers, is a practical impossibility. There is reason to believe that with continued control of epidemic typhoid fever, the reduction in the proportion

of carriers may well be reflected in a reduced incidence of the disease in the endemic form.

Pathogenicity for Lower Animals. The injection of typhoid bacilli into experimental animals produces much the same effect as the injection of colon bacilli. When introduced into the peritoneal cavity in considerable quantity many strains produce symptoms of a non-specific character and a fatal outcome. Although a genuine but slight multiplication of the bacilli takes place and attests the occurrence of a true infection, neither the symptoms nor the lesions of this intraperitoneal typhoid bear any close resem-
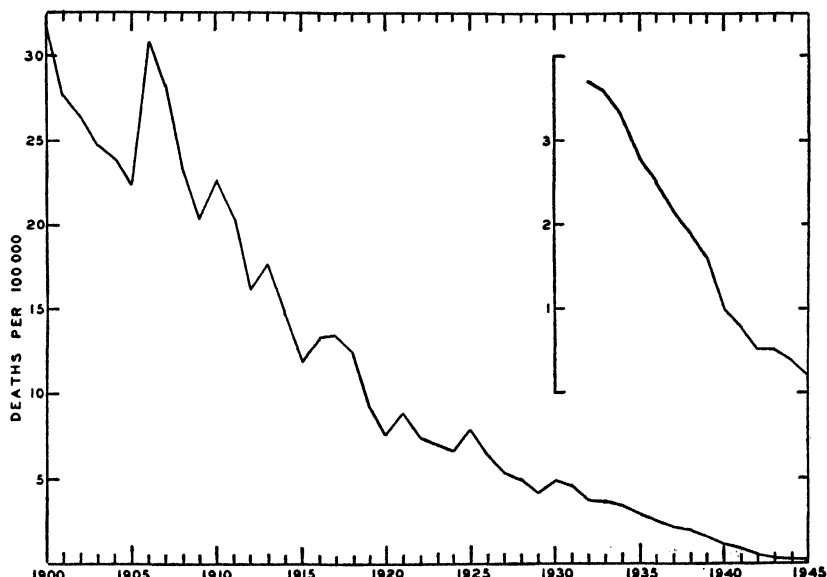
Fig. 77. The prevalence of typhoid and paratyphoid fever in the Registration Area since 1900 as indicated by the uncorrected death rates. From data compiled by the Bureau of the Census.

blance to the typhoidal processes in man. The virulence of the typhoid bacillus for mice on intraperitoneal inoculation is raised to a very high level, only a few hundred sufficing to produce a fatal infection, by suspension in 5 per cent mucin (see Army studies below). Remlinger,[24] however, was able to set up an infection by feeding vegetables smeared with typhoid bacilli to fasting rabbits and rats, but without the reproduction of the clinical picture of human typhoid. Others have reported the reproduction of characteristic typhoid lesions in the ileum in chimpanzees fed typhoid cultures in milk and broth. It may be noted that the carrier state may be produced in the rabbit by intravenous inoculation, the bacilli localizing in the gallbladder, where they may persist for several months. It must be admitted, however, that typhoid fever as it exists in man has never been reproduced in the experimental animal.

[24] Remlinger: Ann. Inst. Pasteur, 1897, *11*:829.

Immunity. An attack of typhoid fever confers a certain degree of immunity, although instances of two or even more attacks in the same individual are not unknown. Animal experiment has shown that it is possible to obtain a high degree of immunity in rabbits and guinea pigs against intraperitoneal inoculation. The immunity is associated with the development of humoral antibodies such as agglutinins, precipitins and the like. Lysin is also produced, and, like the cholera vibrio, the typhoid bacillus undergoes visible dissolution and disintegration in the peritoneal cavity of the immune animal.

In man recovery from typhoid fever is also accompanied by the appearance of demonstrable antibodies. In most instances agglutinins appear during the course of the disease, sometimes as early as the fifth day (over 90 per cent by the fourth week), and their presence is the basis of the Widal test used for diagnostic purposes.

In its original form the Widal test was a slide agglutination test, and agglutination of typhoid bacilli by patient's serum in a dilution of 1:50 or more was considered positive. The development of knowledge of the antigenic structure of the typhoid and paratyphoid bacilli in recent years has resulted in a somewhat better understanding of the value and limitations of the agglutination test in the diagnosis of typhoid fever. At the present time the test is a macroscopic one and is carried out with both H and O antigens. The interpretation of a single such test must take into consideration ancillary data such as a previous immunization or attack of typhoid fever, the prevalence of endemic typhoid in the general population, etc. It is, therefore, difficult to set arbitrary limits; in most instances an O titer of 1:100 and an H titer of 1:200 may be regarded as significant. A point of some interest is the lack of sharp antigenic specificity of human sera as compared with the specificity of experimentally produced rabbit antisera.

The interpretation of the Widal test in immunized persons is often difficult, since both H and O agglutinins are formed in response to the vaccine. Titers fall after immunization, of course, but may persist at moderate levels for many months. Furthermore, the agglutinin titer may rise in anamnestic response to a febrile condition. The extent to which this occurs is not definitely known and some data suggest that it is significant while others do not. Such an anamnestic reaction is particularly prone to occur in typhus fever, and typhoid agglutinin titers as high as 1:800 have been observed.[25] There is some evidence that an "agglutinin curve," obtained by periodic agglutinin titrations, has diagnostic value in that it continues to rise in typhoid fever but usually does not do so in the anamnestic reaction.

Prophylactic Inoculation. Man may be actively immunized against typhoid fever by the parenteral inoculation of killed typhoid bacilli. Antityphoid vaccination has had its widest application thus far in the protection of soldiers in the field, for, owing to the conditions under which they must live in the field or on the march, the likelihood of typhoid fever is great. Mass immunization has effected a remarkable reduction in the incidence of typhoid fever among these men. For example, in 1898 at the time of the Spanish-American War, 4422 cases of typhoid and 248 deaths occurred in a divison of 10,759 men; among 12,801 vaccinated men under very similar

[25] Süpfle: Arch. f. Hyg. u. Bakt., 1943, 129:158.

conditions during summer maneuvers at San Antonio, Texas, in 1911, only one case developed. Similar results have been obtained in the armies of other nations, and the efficacy of typhoid immunization is undoubted. Typhoid vaccination has been compulsory in the United States Army since 1911, and the result has been the practical disappearance of the disease.[26] The immunity so developed is not absolute, of course, and may be broken down by large doses of typhoid bacilli; typhoid fever in immunized personnel of armies is observed from time to time.[27]

The vaccine ordinarily consists of a saline suspension of killed bacteria. The microorganisms are grown on the surface of agar culture media and after eighteen hours' incubation are washed off with sterile physiological salt solution and killed by heating to 55° to 56° C. for one hour. The suspension is standardized by counting the number of bacteria and then diluting so that 1 ml. contains 1 billion bacilli. A preservative (0.25 per cent tricresol) is added and the sterility rigidly controlled by culture and animal inoculation.

The vaccine is given in three injections, five days to a week apart. The first consists of 0.5 ml. or 500 million bacilli, and the second and third 1 ml. or 1 billion bacilli each. As a rule, the reaction following inoculation is not severe, although fever, chills, nausea and nervous symptoms may be observed. At times it has been found advantageous to immunize simultaneously against the paratyphoid infections as well. Such vaccines, known as TAB vaccines (typhoid, para A and para B), are standardized to contain 1 billion typhoid bacilli, 250 million paratyphoid A bacilli and 250 million paratyphoid B bacilli per ml. Under exceptional circumstances still other microorganisms have been added to typhoid vaccines.

While the effective immunogenic potency of typhoid vaccines can, in the last analysis, be measured only by field trial, protection against the experimental infection of mice with bacilli suspended in mucin has been useful. The active and passive mouse protection tests have been used by workers in the United States Army Medical School in extensive studies directed toward improvement of the immunizing antigen.[28] It is reasonably well established that antibody to the O antigen is protective while that to the H antigen is not, and it is also clear that antibody to Vi antigen is protective against infection with Vi-containing bacteria. In general, then, the vaccine should contain adequate amounts of undenatured O antigen and its immunizing potency may also be reinforced by the presence of Vi antigen since most strains of typhoid bacilli isolated from human infections contain it. The Army investigations have shown that highly immunogenic strains of the typhoid bacillus are highly virulent and conclude that such strains should be used for vaccine preparation. The classic Rawlings strain was found to be inferior as assayed by mouse protection and a highly virulent, Vi-containing strain, No. 58, has been substituted for it in Army vaccine. Subsequent

---

[26] The Army experience to 1942 with prophylactic inoculation has been summarized by Callender and Luippold: Jour. Amer. Med. Assn., 1943, *123*:319.

[27] For experience in World War II see, for example, Jordan and Jones: Lancet, 1945, *ii*:333; and Syverton *et al.*, Jour. Amer. Med. Assn., 1946, *131*:507.

[28] These studies are described in detail in a monograph by Siler *et al.*: *Immunization to Typhoid Fever.* Johns Hopkins Press, Baltimore. 1941. Additional studies are summarized by Longfellow and Luippold: Amer. Jour. Pub. Health, 1943, *33*:561.

work[29] has suggested that the vaccine may be further improved by fortification with Vi-containing extracts but such possibilities are still in an experimental stage. Presumably results based on mouse assay may be applied to man but as yet there is no definite evidence that vaccine prepared with strain No. 58 confers a more effective prophylactic immunity in man than Rawlings strain vaccines.

At the present time typhoid vaccines prepared in the United States must conform to an immunogenic potency standard based on the active mouse protection test. "Each of 30 or more mice of any susceptible strain, 6–8 weeks old and weighing 14–16 gms., is given 0.5 ml. of a 1:10 dilution of the vaccine intraperitoneally. Equal numbers of male and female mice should be used in each group. Fourteen days after the injection of vaccine the mice are divided into three groups of not less than 10 mice each, one group to receive
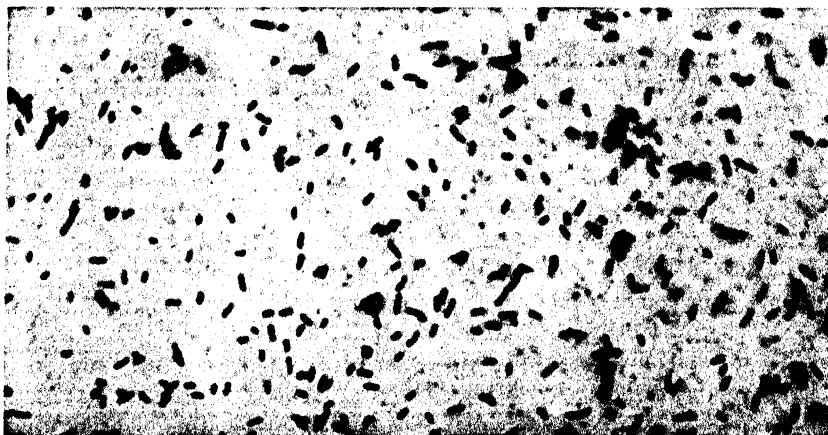
Fig. 78. *Alcaligenes fecalis.* Smear from a pure culture. Fuchsin; × 1050.

approximately 100,000, one group approximately 10,000 and the third group approximately 1,000 lethal doses of virulent typhoid bacilli (16–20 hours old) suspended in 5 per cent mucin." At least 50 per cent of the mice should be protected against not less than 10,000 lethal doses of organisms.

Another type of antigen, consisting of a formalized solution of the cell substance of typhoid bacilli and designated endotoxoid vaccine, has been used in recent years with encouraging results,[30] but it is not as yet clear how this antigen compares with whole bacilli.

As indicated above, typhoid vaccines are ordinarily administered parenterally, by subcutaneous injection. It has been contended by some workers that oral administration of the vaccine together with a small quantity of bile, which presumably increases the permeability of the intestinal wall, produces an equally effective immunity. As pointed out in an earlier chapter, oral vaccination is a highly uncertain method of inoculation having no

[29] Luippold: Amer. Jour. Pub. Health, 1946, 36:15.
[30] Grasset: Brit. Med. Jour., 1939, ii:58; *ibid.,* Pub. South African Inst. Med. Res., 1945, 9:163. See also Morgan, Favorite and Horneff: Jour. Immunol., 1943, 46:301.

advantages over parenteral injection. Vaccination by the oral route is seldom practiced.

Passive Immunization. The use of antityphoid serum for therapeutic purposes has been considered by a number of workers, but there is still no conclusive evidence as to its value. Whether or not "antiendotoxic" sera or other typhoid antisera confer a passive immunity to typhoid infection in man is likewise not established.

ALCALIGENES FECALIS

Alcaligenes fecalis or *Bacterium fecalis alcaligenes* closely resembles the typhoid bacillus morphologically, culturally and even in its growth on Endo, Conradi-Drigalski and malachite green differential media. It has been found in feces and in water. It differs from the typhoid bacillus in the possession of one or more polar, instead of many peritrichous, flagella; more luxuriant growth on potato with a brown coloration; and distinct alkali production in mannitol and litmus milk. It fails to produce acid from dextrose and other carbohydrates. It has been suggested that *Bact. alcaligenes* is a form of *Bact. fluorescens non-liquefaciens* which has lost the function of pigmentation, and it does have affinities with certain of the plant pathogens and soil bacteria. Its systematic position has been examined critically by Conn[31] who has proposed a new genus, *Agrobacterium,* to include this bacillus together with *Bacterium radicicola* and the bacterium of hairy root disease. Other species have been described as occurring in the intestinal tract, *viz., Alcaligenes metalcaligenes, Alcaligenes hookeri* and *Alcaligenes recti,* but these are not ordinarily differentiated from *Alcaligenes fecalis.* Other varieties, *Alcaligenes viscosus* and *Alcaligenes marshallii,* are found in dairy products and produce a slimy alkalinity and ropiness in milk.

Alcaligenes fecalis is only feebly pathogenic for experimental animals and presumably also for man. Human infection is observed in rare instances, however, in the form of mild typhoid-like disease, bacteremia and cystitis.

[31] Conn: Jour. Bact., 1942, 44:353.

THE ENTERIC BACILLI: THE DYSENTERY BACILLI[1]

Dysentery is a clinical rather than an etiological entity, and its characteristic symptoms, diarrhea, abdominal pain and blood in the stools, may occur either alone or as part of the syndrome of a number of diseases. In the former instance dysentery may be of protozoan (amebic dysentery, p. 755) or bacterial etiology. In addition there is evidence suggesting that a filterable agent, presumably a virus, can cause dysentery in man.[2] A dysentery-like infection may be produced by some members of the Salmonella group, but usually another group of bacteria, the dysentery bacilli, is responsible.

The dysentery bacilli are gram-negative, non-spore-forming rods related to the other enteric bacteria. Some of them resemble *Bact. coli anaerogenes* and the typhoid bacillus in that they ferment carbohydrates with the production of acid and no gas. Others are, perhaps, related to the slow or late lactose-fermenters designated as paracolon bacilli. None of the dysentery bacili are motile, and hence they do not contain the two types of antigens found in the paratyphoid bacilli. As a group they differ from one another biochemically and immunologically. In general, their uncertain relationship to the other enteric bacilli, coupled with their own heterogeneity, has made their classification a difficult matter.

The dysentery bacilli are facultative anaerobes and their optimum temperature for growth is 37° C. Their nutritive requirements are not complex in that they will grow upon the ordinary nutrient (beef extract) media. In synthetic solutions nicotinic acid is apparently required by some strains, but whether amino acids are necessary is not known. They ferment glucose to much the same end products as the other enteric forms—lactic acid together with smaller amounts of formic and acetic acids and ethyl alcohol. Like the other gram-negative bacilli, they are relatively resistant to the bacteriostatic action of dyes, and these substances may be incorporated in differential media for their isolation; eosin-methylene blue agar is commonly used.

Classification. The dysentery bacilli are divided into two groups on the basis of the fermentation of mannitol, the non-mannitol fermenters including the Shiga bacillus. This distinction was of early importance and continues to be in tropical regions and elsewhere where the Shiga bacillus occurs, for the dysentery produced by it is much more severe and has a higher case fatality rate than the other bacillary dysenteries, but is not of practical signifi-

[1] See the general reviews by Neter: Bact. Rev., 1942, 6:1; *ibid.*, Gastroenterology, 1943, 1:366; Weil: Jour. Immunol., 1943, 46:13; *ibid.*, 1947, 55:363.

[2] Gordon, Ingraham and Korns: Jour. Exp. Med., 1947, 86:409.

cance in this country where there is little or no Shiga dysentery. In addition to the Shiga bacillus, this group also includes the so-called parashiga bacilli and the Schmitz bacillus. The group of mannitol fermenters is further subdivided on the basis of slow (four to seven days) fermentation of lactose and the fermentation of dulcitol and sorbitol. In all, six groups which are given species rank are distinguished on a biochemical basis.

In the past the nomenclature of the dysentery bacilli has been somewhat casual and informal names such as the Shiga bacillus, the Flexner bacillus, the Strong bacillus, the Hiss-Russell Y bacillus, etc., have had wide currency. The name Shigella for the genus has gained reasonably wide acceptance and the dysentery bacilli are regarded by most workers as species of this single genus. The Bergey (1948) classification dispenses with all the personal names, except in the case of the Sonne bacillus, that have long been associated with these bacteria; this makes for some confusion since only in the United States has this classification met with some degree of general acceptance. For present purposes the following will be regarded as species of the dysentery bacilli:

    (1) Non-mannitol fermenters
            Shigella shigae
            Shigella parashigae—including serological types
            Shigella ambigua (Schmitz)
    (2) Mannitol fermenters
       (a) Non-lactose fermenters
            Shigella flexneri—including serological types
            Shigella alkalescens
       (b) Slow lactose fermenters
            Shigella sonnei
            Shigella dispar

Some of these are immunologically homogeneous and some heterogeneous, and some have been divided into immunological types, as indicated, or biochemical variants.

DIFFERENTIAL BIOCHEMICAL REACTIONS OF THE DYSENTERY BACILLI

Species	Man-nitol	Lactose	Sucrose	Dulcitol	Sorbitol	Rham-nose	Indol
Shigella shigae	−	−	−	−	−	−	−
Shigella parashigae	−	−	−	−	−	−	−
Shigella ambigua	−	−	−	−	+	+	+
Shigella flexneri	+	−	±	∓	±	∓	±
Shigella alkalescens	+	−	±	+	+	−	+
Shigella sonnei	+	+(1)	+(1)	−	−	+	−
Shigella dispar var. *ceylonensis*	+	+(1)	+(1)	+	+	+	+
Shigella dispar var. *madampensis*	+	+(1)	+(1)	−	+	+	+

± most strains ferment; ∓ most strains do not ferment.

Shigella Shigae (The Shiga Bacillus, *Bacterium dysenteriae*, *Shigella dysenteriae*). This was the first of the dysentery bacilli to be described.

The Japanese bacteriologist, Shiga, found the bacterium which bears his name in a study of an epidemic of dysentery in Japan in 1898. The same microorganism was found by Kruse in Germany two years later, and *Shigella shigae* is known as Shiga's bacillus or the Shiga-Kruse bacillus. The Shiga bacilli are immunologically homogeneous, but anti-Shiga sera show some cross-reaction with some strains of *Shigella flexneri* and *Shigella ambigua*.

Sh. shigae differs from the other dysentery bacilli in its marked toxicity for man and experimental animals. Apparently two types of toxin are formed; an endotoxin closely bound to the cell substance and which is a polysaccharide-lipid-polypeptide complex[3] that appears to have considerable immunizing activity as assayed by mouse protection, and an exotoxin found in filtrates of broth cultures, protein in nature and thermolabile. The exotoxin (neurotoxin) has an effect upon the nervous system and produces paralysis, while the endotoxin appears to act chiefly upon the alimentary tract. Although generally regarded as a true soluble toxin, the Shiga toxin is not as potent as the toxins of the diphtheria and tetanus bacilli; it has been prepared in purified form having an LD_{50} dose for mice of 1 to 10 μg.[4] Antitoxin to this soluble toxin may be produced, though not to the high titers obtained against other soluble toxins, which has marked protective effect in animal experiments. The therapeutic use of these sera has given encouraging results in some instances, but their value is not firmly established. The soluble toxin, but not the endotoxin, may be inactivated by formaldehyde and the toxoid used as an immunizing agent. It has not yet been possible to destroy the toxicity of this or other dysentery bacillus endotoxins and at the same time retain antigenicity.

Shiga bacillus infections have been observed most frequently in India, Japan, China and other parts of Asia; they appear to be relatively rare in the United States.

Shigella Parashigae. Strains of dysentery bacilli culturally identical with *Sh. shigae* but immunologically unrelated were found by Dudgeon and Urquhart[5] in Macedonia in 1919 and designated by them *Bacterium parashigae* (—) in contrast to the Schmitz bacillus (see below) which they termed *Bacterium parashigae* (+). These bacilli have been observed from time to time in various parts of the world, including the United States, associated with diarrheal disease. They were studied in some detail by Large[6] and by Sachs[7] and are sometimes known as the Large-Sachs group or Sachs group of dysentery bacilli. Sachs distinguished eight immunological types but Wheeler and Stuart[8] found that three of these were paracolon bacilli, and described an additional new type, making six valid types in all. The Sachs types are Q454, Q771, Q902, Q1030 and Q1167 and the Wheeler and Stuart type 1831.

Other non-mannitol fermenting dysentery bacilli which differ immuno-

[3] Morgan and Partridge: Biochem. Jour., 1940, 34:169.
[4] Dubos and Geiger: Jour. Exp. Med., 1946, 84:143.
[5] Dudgeon and Urquhart: Med. Res. Council Spec. Rept. Series No. 40, 1919.
[6] Large: Jour. Roy. Army Med. Corps, 1934, 63:80, 231.
[7] Sachs: Jour. Roy. Army Med. Corps, 1943, 80:92.
[8] Wheeler and Stuart: Jour. Bact., 1946, 51:317.

logically from *Sh. shigae* have been described. Of these the organism desig-
nated *Sh. arabinotarda* type A and Gober and Stacy strain 8524 has been
found to be identical with Q771, and *Sh. arabinotarda* type B with Q1167.
Still another, *Sh. wakefield,* is a paracolon bacillus. At the present time,
then, *Sh. parashigae* appears to be immunologically heterogeneous and made
up of six types which may be designated Type Q454, Type Q771, etc.

Shigella Ambigua (Schmitz bacillus, *Bacterium schmitzii, Bacterium
ambiguum*). This species was described by Schmitz in 1917 as a cause of
dysentery in a Rumanian war prison camp. Like *Sh. shigae,* it does not fer-
ment mannitol but differs in that it produces indol and ferments sorbitol and
rhamnose. The species is immunologically homogeneous except that, accord-
ing to Boyd,[9] freshly isolated strains contain two antigens, one of which is
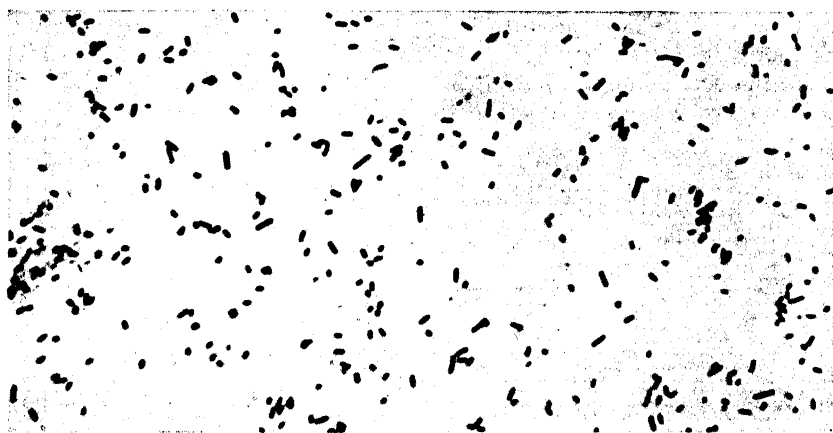
Fig. 79. *Shigella flexneri.* Smear from a pure culture. Fuchsin; × 1050.

lost on continued cultivation, and antisera prepared from stock strains may not
agglutinate fresh strains too well. There is some cross reaction, perhaps to one-
quarter titer, with the Shiga bacillus but agglutinins are not reciprocally ab-
sorbed. *Sh. ambigua* has been found in Europe, India, the Sudan and else-
where, and in the United States. It is not as common in this country as some
of the other dysentery bacilli but is encountered with some frequency and was
implicated in an extensive institutional outbreak of dysentery in New York[10]
and as an important cause of dysentery among chimpanzees.[11]

Shigella Flexneri (*Bacterium paradysenteriae,* Pseudodysentery bacillus,
Shigella paradysenteriae, Flexner's Bacillus, Hiss and Russell's "Y" Bacillus,
Strong's bacillus). Soon after Shiga's discovery, Flexner, working in the
Philippines, discovered other dysentery bacilli which for a time were not
clearly differentiated. Flexner's bacilli and those described by Strong and
Musgrave in 1900 differed from *Sh. shigae,* however, both serologically and
in the fermentation of mannitol. Attempts to subdivide the bacilli of the
Flexner group by biochemical methods have not been successful; a wide

[9] Boyd: Jour. Roy. Army Med. Corps, 1935, 64:289.
[10] Schliefstein and Coleman: Jour. Inf. Dis., 1937, 61:257.
[11] Galton, Mitchell, Clark and Riesen: Jour. Inf. Dis., 1948, 83:147.

variety may be separated on the basis of the variable fermentation of sucrose, dulcitol, sorbitol, maltose, raffinose, arabinose, inositol and salicin, and indol formation, but such varieties are not correlated with immunological type and have had little practical value.

Sh. flexneri is made up of a group of immunological types that are distinct and yet related to one another. Five immunological types were distinguished by Andrewes and Inman[12] on the basis of the distribution of four antigens, V, W, X and Z, which are designated as types V, W, X, Y and Z and whose antigenic composition is illustrated in Fig. 80. A number of other systems of typing *Sh. flexneri* were suggested but none gained the general acceptance of the Andrewes and Inman types.

Subsequently Boyd[13] reported evidence of the presence of type- and group-specific antigens in these types and in additional related forms and suggested that numbered types be substituted for the Andrewes and Inman types. Further study by Wheeler[14] indicated the pattern of distribution of antigens among the types suggested by Boyd with the modification that Type II be split into two subtypes; this is illustrated in the accompanying table. The
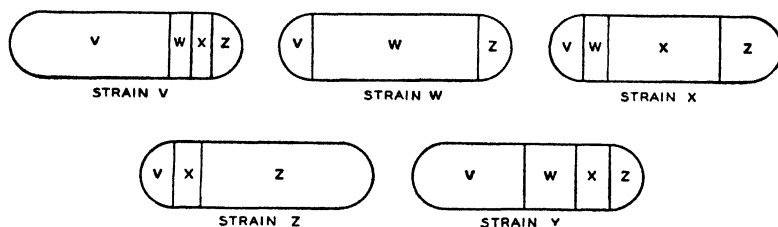
Fig. 80. Diagrammatic representation of the antigenic structure of the Andrewes and Inman types of *Sh. flexneri*.

basis of Boyd's contention that the O and Y types of Andrewes and Inman are not valid since they contain no type-specific antigen and are possibly to be regarded as degraded variants of Types IIa and IIb is clear from this. Of the Andrewes and Inman types accepted by these workers, V, W, and Z become Types I, II and III respectively. Types IV and V are new types, first found by Boyd in India and called by him 103 and P119 respectively.

Type VI, or Boyd 88, is not new but a variety of the Newcastle-Manchester bacillus which was first isolated in 1929 from cases of dysentery.[15] The original Newcastle strain appears to be somewhat apart from the other dysentery bacilli in that it is feebly motile and produces gas, usually in small amount, in the fermentation of glucose. The original Newcastle strain did not ferment mannitol, but the variety known as the Manchester type does ferment this alcohol; some strains produce a small amount of gas and others do not. Almost all of the Newcastle-Manchester bacilli isolated in the United States have been of the mannitol-fermenting, non-gas-forming type. The variety known as Boyd 88 ferments mannitol but does not form gas, and about one-third of the strains

[12] Andrewes and Inman: Med. Res. Council Spec. Rept. Series No. 42, 1919.
[13] Boyd: Jour. Hyg., 1938, 38:477; Trans. Roy. Soc. Trop. Med. Hyg., 1940, 33:553.
[14] Wheeler: Jour. Immunol., 1944, 48:87.
[15] Clayton and Warren: Jour. Hyg., 1929, 28:355; *ibid.*, 1929, 29:191.

do not ferment dulcitol, and the remainder are late dulcitol fermenters. The Newcastle-Manchester bacilli are all late dulcitol fermenters and produce a small amount of gas. The group is, however, immunologically homogeneous and related to the Flexner bacilli and may not unreasonably be regarded as a type of *Sh. flexneri.*

ANTIGENIC STRUCTURE OF *SH. FLEXNERI* TYPES*

Andrewes and Inman and Boyd Types	Antigenic Structure		Type
	Type Specific	Group Specific	
V	I	1, 2, 4, 5, 6, 9	I
W	II	1, 3, 4	IIa
	II	1, 7, 8, 9	IIb
X	none	1, 7, 8, 9	
Y	none	1, 3, 4	
Z	III	1, 6, 7, 8, 9	III
103	IV	1, 6	IV
P119	V	1, 5, 7, 9	V
88	VI	1, 2, 4	VI

* According to Wheeler: Jour. Immunol., 1944, *48*:87.

Other mannitol-fermenting dysentery bacilli have also been described by Boyd as 170, P288, D1, D19, P143 and P274. These are not related immunologically to the Flexner bacilli, and it has been suggested that they be named *Sh. boyd* Types I, II, III, IV, V and VI respectively. They are, however, included by Weil, Black and Farsetta[16] with the Flexner dysentery bacilli. These workers accept Types I to VI of *Sh. flexneri* as defined by Boyd but add the Andrewes and Inman types X and Y as Types VII and VIII. The Boyd types are then added to the series as Types IX to XIV inclusive; Type IX is 170 or Boyd I, Type X is P288 or Boyd II and so on.

Still other mannitol-fermenting dysentery bacilli giving other biochemical reactions typical of *Sh. flexneri,* but immunologically unrelated, have been included in this species. A variety found in the Mediterranean area and named *Sh. etousae* has been included as *Sh. flexneri* Lavington I, and Francis,[17] disregarding Weil's numbering of types, has suggested that two varieties immunologically related to the Andrewes and Inman types be provisionally typed as Types VII and VIII.

It seems clear that this somewhat confused state stems from the lack of a reasonably precise, generally accepted, working definition of *Sh. flexneri.* If it be made on a purely biochemical basis, the extended series of types of Weil, Black and Farsetta should logically stand, to be amplified as other immunological varieties of the biochemical type are described. If, however, the definition of the species is also to call for an immunological interrelationship, only

16 Weil, Black and Farsetta: Jour. Immunol., 1944, 49:321.
17 Francis: Jour. Path. Bact., 1946, 58:320.

Types I to VI are valid. The present tendency, which is not completely consistent, is to continue to accept the Andrewes and Inman types and regard the Newcastle-Manchester bacilli as somewhat apart though immunologically related, or to accept the first six types only. As for the remainder, only the British as yet recognize *Sh. boyd* Types I to VI, and in this country these are designated *Shigella sp.* 170, *Shigella sp.* P288 and so on.

All of the Flexner bacilli are of world-wide distribution, the relative proportions varying from one locality to another. As a whole *Sh. flexneri* is found in a large proportion of the dysentery cases in temperate climates, and even in tropical countries they are perhaps the most common of the dysentery bacilli. In this country, for example, 451 strains of a total of 769 isolated in routine examinations in Connecticut in 1940–43 were *Sh. flexneri*, and of 1329 dysentery bacilli isolated from British and Indian troops in India, 999 were Flexner, 197 Shiga, 100 Schmitz and 33 Sonne bacilli.
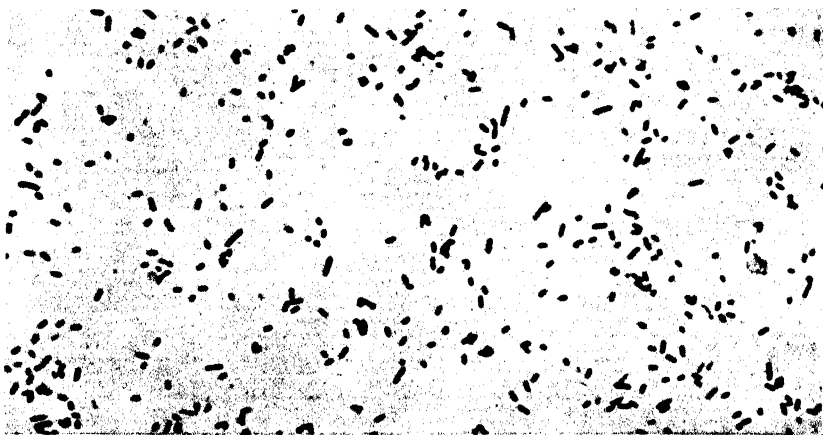
Fig. 81. *Shigella alkalescens.* Smear from a pure culture. Fuchsin; × 1050.

Sh. flexneri forms no soluble or exotoxin but does contain an endotoxin. It has been studied in some detail by Goebel and his co-workers[18] who have found that the somatic antigen which contains the toxicity is made up of a lipid component, a protein component, two carbohydrate components one of which contains labile acetyl groups, and a toxic component which Goebel believes to be a distinct substance and possibly associated with a purine or pyrimidine-like substance. In general, it has not been possible to detoxify the endotoxin without destroying its antigenicity, though Barnes *et al.*[19] have reported that this can be accomplished by treatment with heat or ultraviolet light in the presence of an oxidizing agent and caprylate ion.

Shigella Alkalescens. *Shigella alkalescens* was described by Andrewes in 1918. Unlike the other dysentery bacilli, these bacilli ferment dulcitol. For some time they have been regarded as of uncertain pathogenicity but evidence is accumulating which indicates that they are associated with disease. They

[18] Goebel *et al.*: Jour. Bact., 1944, 47:476; Jour. Exp. Med., 1945, 81:315, 331; *ibid.*, 1947, 85:499.

[19] Barnes *et al.*: Jour. Immunol., 1947, 56:255.

have been found associated with sporadic enteric disease, and a small epidemic has been reported recently.[20] In the last few years *Sh. alkalescens* has been recognized more and more frequently in this country, and appears to be more common than had been supposed. It is also pathogenic for experimental animals.[21]

Strains of *Sh. alkalescens* have been regarded as biochemically distinct and immunologically homogeneous. Stuart, Rustigan, Zimmermann and Corrigan,[22] however, have found that the species is made up of a graded series of biochemical types. They have also demonstrated the presence of five antigens, two major antigens designated A and B, and three minor antigens, C, D and E, in these bacilli. A, B and C are present in all typical strains, while D and E occur singly or in combination to give four subtypes. They are immunologically related to the colon bacilli through the paracolon group and to Boyd P274. Two immunologically unrelated types have been described[23] and designated type II and type III in distinction to the original or type I. Weil and Slafkovsky[24] have suggested that type II be *Shigella tieté*. Both of these types include strains which ferment lactose. Whether these should be classified as *Sh. alkalescens* is possibly open to some question.

Shigella Sonnei (Sonne's Group III, Duval's bacillus). Lactose-fermenting dysentery bacilli discovered by Duval in 1904 have since been rediscovered by quite a number of observers. By force of usage these bacteria have come to be known as the Sonne type. The Sonne bacillus ferments mannitol and does not produce indol; it is serologically distinctive and homogeneous. Glynn and Starkey[25] have reported, however, that there are two immunological types of *Sh. sonnei* which they designate I and II. Type I contains one antigen in predominance, while type II contains both antigens in equal amounts. The difference in agglutination titers is of practical importance and type II antiserum is the one of choice. The lactose fermentation is slow and may be delayed for a week or ten days, and strains of this type were doubtless confounded with Flexner bacilli by the earlier workers. It is probably considerably more common in the United States than appears in the records. It has also been reported in warmer countries, and perhaps a more detailed study of the differentiation of the mannite-fermenting bacilli would increase the amount of dysentery properly ascribable to this microorganism.

The slow lactose fermentation would appear to relate *Sh. sonnei* to members of the colon group and in particular the so-called paracolon bacilli, but their immunological homogeneity tends to set them off.

Shigella Dispar. Other lactose-fermenting dysentery bacilli were designated *Bact. dispar* by Andrewes. Some of Andrewes' strains were *Sh. sonnei*, but the type now termed dispar differs from the Sonne type in that sorbitol is fermented and indol is formed. *Sh. dispar* is serologically heterogeneous

[20] Felsen and Wolarsky: New York State Jour. Med., 1940, 40:1303; Stuart *et al.*: Jour. Immunol., 1943, 47:425.
[21] Edward: Jour. Path. Bact., 1940, 51:245.
[22] Stuart, Rustigan, Zimmermann and Corrigan: Jour. Immunol., 1943, 47:425.
[23] Assis: O Hospital, 1939, 15:447, 655; Neter: Proc. Soc. Exp. Biol. Med., 1944, 57:200.
[24] Weil and Slafkovsky: Jour. Bact., 1948, 55:759.
[25] Glynn and Starkey: Jour. Bact., 1939, 37:315.

and not related to *Sh. sonnei* but appears to be related to certain Flex-ner strains. Carpenter[26] found that a group of 37 strains could be sep-arated into three immunological types, two related to one another and the third independent. *Sh. dispar* may be divided into two varieties, *Sh. dispar* var. *ceylonensis* which ferments dulcitol, and *Sh. dispar* var. *madampensis* which does not; Bergey (1948) gives these varieties species rank as *Sh. ceylonensis* and *Sh. madampensis*.

Pathogenicity for Man. The serum of patients suffering from acute dysentery agglutinates one or another type of dysentery bacillus in high dilu-tions. This fact and the constant occurrence of the same type of bacillus in the stools strongly suggest a causal relation in spite of the fact that the dysentery bacilli cannot, like the typhoid bacillus, be cultivated from the blood of the patients. Laboratory infection with a pure culture of the Flexner bacillus has occurred.[27]

The incubation period of bacillary dysentery is generally short, about forty-eight hours, and the disease may be acute or tend to run a chronic course. Apart from the inflammatory, sometimes ulcerative or diphtheritic, lesions in the intestine (ulcerative colitis), the anatomical picture of dysentery presents little that is characteristic. The liver abscesses that are found, as a rule, in amebic dysentery are absent in the bacterial diseases, one series having been reported of 1130 cases of bacillary dysentery without a single abscess. Dysen-tery bacilli are sometimes found in immense numbers in the dejecta, often in almost pure culture. They may be found at autopsy in the mesenteric glands, but, as a rule, not in the spleen or other internal organs, nor do they com-monly occur in the blood or urine. Bacillary dysentery is, therefore, not a septicemia but an infection localized in the alimentary tract, in this respect resembling Asiatic cholera rather than typhoid fever. Recurrent diarrheal disease, often called chronic ulcerative colitis, is frequently caused by dysentery bacilli, indicating that the bacilli may persist in the bowel, possibly in the superficial layers of the intestinal epithelium, for long periods of time.

The dysentery toxin is excreted in rabbits, and probably in man, by the large intestine. The selective action of the toxin upon the tissues, rather than any local action of the bacilli themselves, appears to be responsible for the inflammation and other local changes. When the toxin is introduced directly into the gut, no symptoms are produced, suggesting that the toxin primarily affects the deeper cells rather than the surface of the mucous membrane.

In the large series of cases studied in Denmark caused by the Sonne and Flexner types the case fatality was about 2 per cent. The Shiga bacillus dysen-tery of the tropics is much more often fatal (20 per cent). The clinical disease is considerably more severe in the Shiga infections than in Flexner infections, and complications, such as arthritis, are almost invariably associated with the former type of infection.

The relation of dysentery bacilli to summer diarrhea of infants is obscure. Some investigators have isolated the dysentery bacilli from the excreta, par-ticularly in those cases where there is mucus in the stools. Those cases with which dysentery bacilli are associated do not appear to differ clinically from

[26] Carpenter: Proc. Soc. Exp. Biol. Med., 1943, 53:129.
[27] Lippincott: Jour. Amer. Med. Assn., 1925, 85:901.

those in which they are not found, and it is uncertain just what proportion of cases of infant diarrhea is caused by the dysentery bacilli.

Carriers. It is probable that dysentery bacillus infection is very common, many of the infections going unrecognized because of the mildness of the symptoms. Persons with such infections are, of course, convalescent carriers who continue to discharge the bacilli for an average of three to five weeks. It is not clear whether a permanent chronic carrier state analogous to the chronic typhoid bacillus carrier occurs, but many convalescents continue to discharge dysentery bacilli over long periods of time and inapparent infection is not uncommon. In any case, it is the casual carrier and changing groups of casual carriers or ambulatory cases that are of primary importance in the maintenance and spread of the infection.
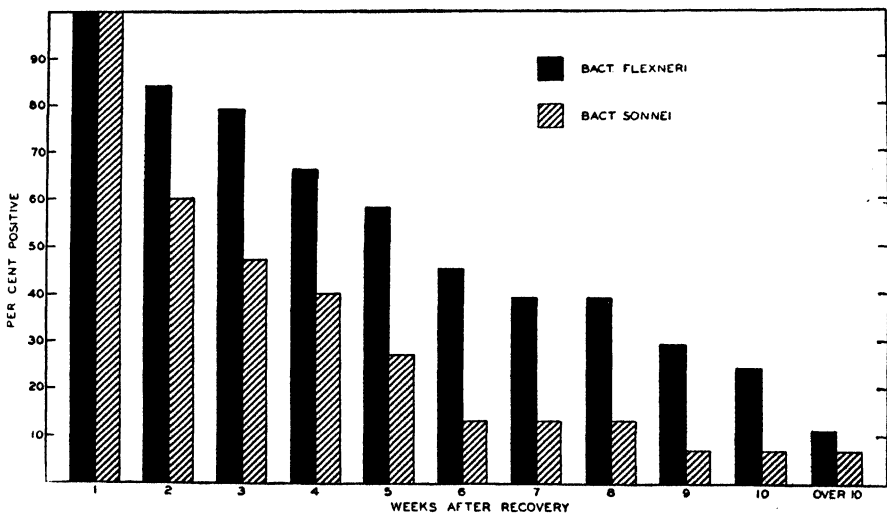
Fig. 82. The persistence of dysentery bacilli in the feces of convalescents. Prepared from data of Watt, Hardy and DeCapito: Pub. Health Repts., 1942, 57:524.

Bacteriological Diagnosis of Bacillary Dysentery. Since, as indicated earlier, dysentery is a clinical rather than an etiological entity, the causative microorganism must be isolated and identified to allow a diagnosis of bacillary dysentery. The bacilli may be found in fecal specimens and rectal swabs are cultured for the detection of carriers. The methods of enrichment culture and direct plating are essentially those used in the isolation of Salmonella and the typhoid bacillus. Desoxycholate-citrate and S-S agars are the most useful of the agar media; bismuth-sulfite is not suitable because species other than *Sh. flexneri* are inhibited. A tellurite-iron-rosalic acid medium has been developed by Wilson and Blair[28] which is said to give good results,[29] but has not as yet been generally used. Colonies are picked and identified by fermentation reactions and slide agglutination; the latter may include typing of the Flexner strains.

[28] Wilson and Blair: Brit. Med. Jour., 1941, *ii*:501.
[29] Thomas and Hulme: Lancet, 1942, *ii*:321.

Epidemiology. Infections caused by dysentery bacilli are probably far more common than is generally recognized. In 1945, 33,495 and 400 deaths were reported from 38 states, rates of 32.4 and 0.4 per 100,000 respectively. Attacks of severe illness grade off into mild and almost trivial attacks of simple diarrhea. Several outbreaks of typical "food poisoning" attributed to the Sonne bacillus are on record. In a number of localities where careful bacteriological studies have been made, dysentery bacilli have been found widely distributed both in patients with gastro-intestinal derangements and in the general population. Probably the most important single reservoir of infection is the human carrier, either convalescent or with inapparent infection. The extent to which the carrier state occurs has been appreciated only in recent years. In a study of dysentery bacillus infection in the normal population Watt and Hardy[30] found *Sh. flexneri* in 11 per cent of the population in New Mexico, 4 per cent

BIOCHEMICAL SEPARATION OF THE DYSENTERY BACILLI
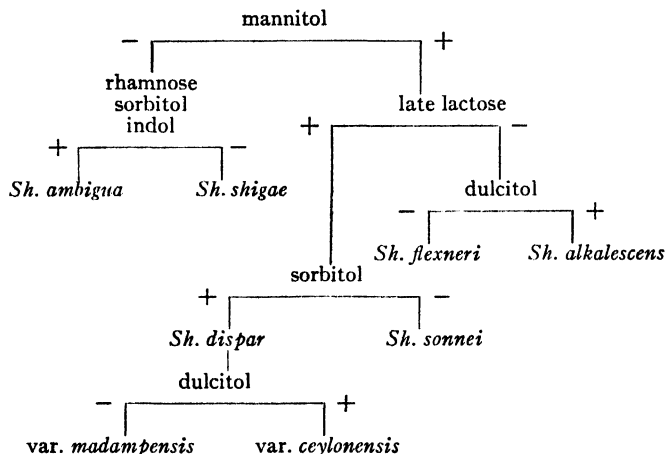
in Puerto Rico, 3 per cent in Georgia and 0.1 per cent in New York City, with an estimated annual morbidity of 60 per cent in Puerto Rico, 48 per cent in New Mexico and 20 per cent in Georgia and an over-all ratio of convalescent or passive carriers to cases of 9.1.

Dysenteric infections seem to be most common in hot countries and in the summer months in temperate climates, although they may occur at any season of the year. The spread of the disease is due to the more or less direct transfer of the specific bacillus from infected intestinal discharges to the alimentary tract of a fresh individual. Polluted water may play a part in some outbreaks but is apparently not nearly so important a factor in dysentery as it is in typhoid fever. Improper disposal of excreta permitting dissemination by flies, and the contamination of food by chronic carriers and convalescents, appear to be the most important factors in the spread of bacillary dysentery. The role of insects, especially flies, is probably an important one and the seasonal incidence of bacillary dysentery is in keeping with this. In the epidemic reported by Kuhns and Anderson infected flies were caught in kitchens and

[30] Watt and Hardy: Pub. Health Repts., 1945, 60:261.

operating latrines; similar reports are not common. The decline in diarrhea and enteritis of the last few decades is very likely a reflection of the general improvement in sanitary conditions.

At the present time in temperate climates dysentery flourishes especially in insane asylums and other large institutions, where lack of personal hygiene among the inmates favors the transfer of infection. In the Denmark investigation endemic asylum dysentery was found to be a serious and prevalent disease. Whenever it gains a foothold in these institutions it seems to be kept alive chiefly by chronic carriers and proves an obstinate problem. Weekly bacteriological examination in one institution showed that more than 50 per cent of the dysentery patients continued to excrete dysentery bacilli for long periods—in one case over four and one-half years. Epidemic bacillary dysentery is also a disease of armies in the field, where opportunities for the dissemination of infection are frequently very great and extensive outbreaks are common.
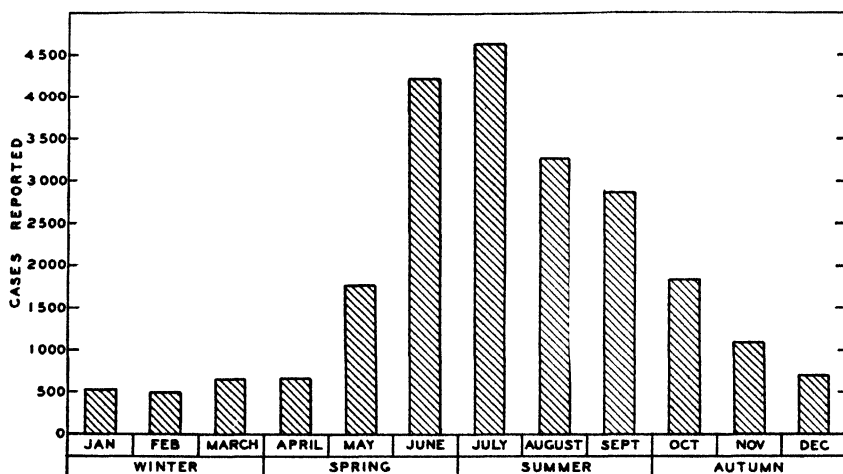
Fig. 83. The seasonal incidence of bacillary dysentery. Averages of reported cases by months for the years 1939 to 1942 inclusive. Data from Supplements to Public Health Reports.

Although in small outbreaks a single type of dysentery bacillus may be found, in the larger outbreaks more than one type is almost always observed. The most common types in this country appear to be Flexner, Sonne, Schmitz and *Sh. alkalescens* in that order.

Immunity. Antibodies, agglutinins, are formed in response to infection with dysentery bacilli, usually appearing after the sixth day. The titer is relatively low as a rule. The diagnostic significance of agglutinins is somewhat uncertain largely because "normal" agglutinins are common. Normal serum commonly agglutinates *Sh. shigae* in 1 : 20 dilution but a titer of 1 : 40 or higher is suggestive of infection. Agglutinins for *Bact. flexneri* occur in much higher titer, as high as 1 : 150 in normal serum, and the titer is frequently increased in infections with other species of dysentery bacilli. Flexner agglutinin has, then, very little diagnostic value unless it is to high titer and agglutinins

for Shiga and typhoid bacilli are absent. Agglutinins to the Newcastle bacillus, *Sh. ambiguum* and *Sh. alkalescens* occur only in low titer. *Sh. sonnei* is often agglutinated to titers as high as 1 : 50 by normal serum, and, though the titer may occasionally be very high in infection, sometimes little or no agglutinin response is apparent.

Despite the immunological response evidenced by the appearance of agglutinins, there is little or no resistance to second attacks and no method of active immunization, other than to the soluble toxin of *Sh. shigae*, has yet proved effective in bacillary dysentery. In this connection it may be recalled that the infection is confined to the lumen of the intestine, intestinal mucosa
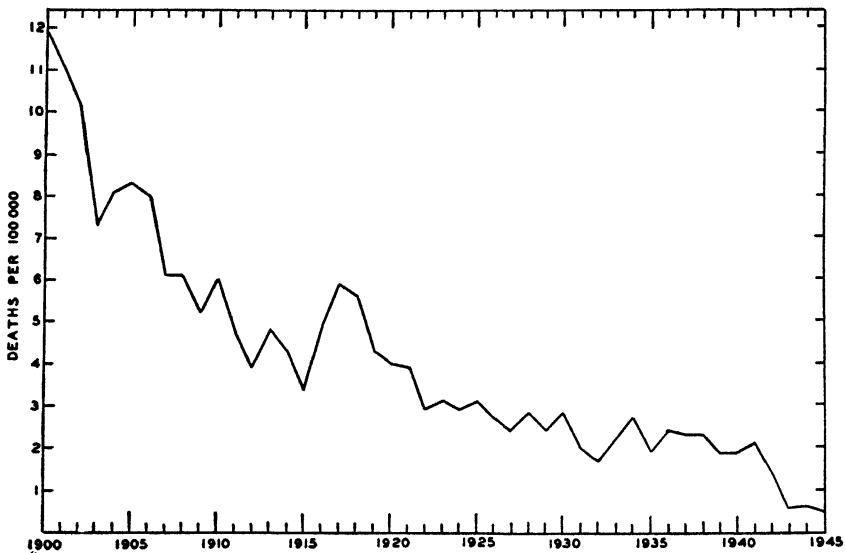
Fig. 84. The prevalence of dysentery in the Registration Area since 1900 as indicated by the uncorrected death rates. From data compiled by the Bureau of the Census.

and lymphoid tissue as a rule and in a very real sense remains outside the body. Bacillary dysentery, then, differs from typhoid fever in the important respect that the bacilli do not invade the tissues and consequently are not exposed to the action of circulating antibody and phagocytic cells. Possibly this accounts, at least in part, for consistent failure to develop an effective immunity to infection with these bacilli.

Pathogenicity for Lower Animals. As pointed out above, the cell substance of the dysentery bacillus is markedly toxic to experimental animals upon parenteral inoculation. The Shiga bacillus is by far the most toxic. Considerable quantities of the other dysentery bacilli are required to elicit symptoms of toxemia. Feeding with dysentery bacilli is generally not successful, though the administration of large doses of living bacilli has resulted in dysenteric symptoms in cats and rhesus monkeys. An experimental infection of the urinary bladder of female guinea pigs has been described by Bingel[31]

[31] Bingel: Ztschr. f. Hyg. u. Infektionskr., 1943, *125*:1.10; *ibid.*, 1944, *125*:574, 610.

in which the local pathology was similar to that found in human bacillary dysentery but there was also an invasion of various tissues and organs. Lower animals apparently do not suffer from bacillary dysentery under natural conditions, although it is not uncommon in the laboratory to isolate *Sh. flexneri* from monkeys that develop enteric symptoms.

THE CHOLERA VIBRIO AND RELATED FORMS

Although Asiatic cholera has doubtless smoldered endemically in parts of India for many centuries, the year 1817 marked its first considerable extension beyond the borders of that country. Europe was first invaded in 1831, and since that date a series of great epidemics has carried the disease over a large part of the civilized world. The disease was brought to New York by Irish immigrants during the pandemic of 1832–33 and in the pandemic of 1846–62 invaded the United States via New Orleans (1848) and spread up the Mississippi Valley. The fourth great pandemic, that of 1864–75, affected Asia, Africa, Europe and America. The causal agent of the disease, the cholera vibrio, was discovered in 1883 by Koch[1] in the intestinal discharges of cholera patients. Similar microorganisms were described by later workers in infected water and elsewhere, and now a number of species are known. In general, however, these other vibrios are nonpathogenic and have been studied in terms of their relation to the cholera vibrio and are, therefore, not particularly well known. Of what is now a fairly sizable group, only two species are pathogenic, the microorganism discovered by Koch and variously termed *Spirillum cholerae asiaticae* (Koch), *Spirillum cholerae, Vibrio cholerae,* the comma bacillus, and *Vibrio comma* (Bergey); and a vibrio pathogenic for pigeons and guinea pigs, *Vibrio metchnikovii.*

VIBRIO CHOLERAE

Morphology and Staining. The cholera vibrio is a short, slightly curved and twisted rod, 1.5 to 3 μ in length and 0.4 to 0.6 μ in breath. It may occur singly or in chains which have the appearance of short spirals or S-shaped forms (two cells). The straight and spiral threads formed in the pellicle of liquid gelatin cultures are usually regarded as involution forms. Cultures that have been maintained for a long time on agar often lose the curved form and appear as straight rods, but resume the more characteristic form when passed through animals. The vibrios are actively motile by a single polar flagellum which is shorter than the flagella of most bacteria. Spores are not formed. The cholera vibrio stains readily with the ordinary aniline dyes and is gram-negative.

Colonies on agar media are similar to those of the other enteric bacilli, but may be distinguished from those of *Bact. coli* by their thin, opalescent appearance. They are 1 to 2 mm. in diameter, low, convex and grayish-yellow in color, with a finely granular consistency which is accentuated under low mag-

[1] Koch: Ber. klin. Wchnschr., 1884, 21:477; Brit. Med. Jour., 1884, ii:403, 453.

nification. Some strains are hemolytic on blood agar while others are not (see below).

Physiology. The cholera vibrio is strongly aerobic and only very sparse growth appears under anaerobic conditions, and then on prolonged incubation. It grows over the temperature range of 16° to 42° C. with an optimum growth
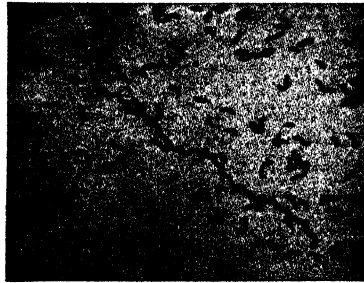
Fig. 85. *Vibrio cholerae;* broth culture two days old; fuchsin stain; × 1000 (Fränkel and Pfeiffer).

at 37° C. An alkaline reaction is essential for good growth; the bacteria will grow over a *p*H range of 6.4 to 9.6 and are usually cultivated at an alkaline reaction, *p*H 7.8 to 8.0. This marked tolerance for alkali is taken advantage of in the preparation of selective media for the isolation of the cholera vibrio, the *p*H of such media usually being about 9.5. They are not nutritionally fastidious and may be grown in peptone water.

THE HEIBERG FERMENTATION TYPES OF VIBRIOS

Sugar	Type					
	I	II	III	IV	V	VI
Sucrose............	+	+	+	+	−	−
Mannose..........	+	−	+	−	+	−
Arabinose..........	−	−	+	+	−	−

Fermentation reactions are variable and a number of carbohydrates, including dextrose, levulose, galactose, maltose, sucrose and mannitol, may be fermented with the production of acid but no gas. Lactose, inulin and dulcitol are not attacked. Heiberg[2] has studied the fermentation reactions of the vibrios at some length and has arrived at six fermentative types on the basis of the fermentation of sucrose, arabinose and mannose which are known as the Heiberg types. Type I is characterized by the fermentation of sucrose and mannose but not arabinose, and contains all of the cholera vibrios and some non-cholera

[2] Heiberg: C. R. Soc. Biol., 1934, 115:984.

varieties. Starch is hydrolyzed. Both coagulated serum and gelatin are liquefied. Stab cultures in gelatin often develop a small turnip-shaped area of liquefaction at the surface, which by evaporation of the fluid leaves a bubble-like depression, while in some growth little or no liquefaction occurs along the needle puncture. Other vibrios besides the cholera vibrio, however, produce this same type of liquefaction. Growth in milk does not produce any visible change for some time, but a slow peptonization without coagulation appears on continued incubation. Hydrogen sulfide and indol are produced and nitrates are reduced to nitrites. The addition of sulfuric acid to a culture of the cholera vibrio in nitrate-peptone broth results in the development of a red color—the so-called "cholera-red reaction." This is, of course, the nitroso-indol reaction and is given by any bacterium, as the colon bacillus for example, that both reduces nitrate and produces indol. Other vibrios give this reaction also.
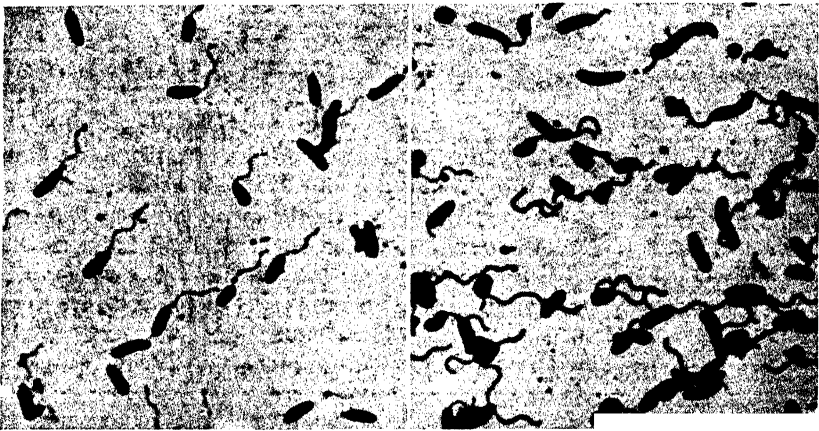
Fig. 86. *Vibrio cholerae* (left) and the vibrio of Finkel and Prior (right), showing the single polar flagella. × 2000 (Kral).

The question of the hemolytic activity of the cholera vibrio has been of considerable importance in relation to its differentiation from the closely related El Tor vibrios. The term hemolysis, when applied to the vibrios, refers to hemolytic activity as determined by the *Greig test* in which a 3 per cent suspension of goat erythrocytes is mixed with an equal quantity of twenty-four-hour broth culture of the vibrio and read after two and four hours' incubation at 37° C. By this test the cholera vibrio is non-hemolytic.[3] As indicated above, however, some strains of the cholera vibrio are hemolytic on blood agar. The apparent contradiction has been resolved by van Loghem[4] in particular, who has shown that in the case of this bacterium at least, blood plate hemolysis is a hemodigestive process and basically different from the liberation of hemoglobin from suspensions of erythrocytes.

The resistance of the cholera vibrio to various injurious influences is not great. It is killed by moderately high temperatures (ten minutes at 55° C.) and is destroyed quickly by chemical disinfectants. It is particularly sensitive

[3] For a recent study see Read, Pandit and Das: Indian Jour. Med. Res., 1942, 30:183.
[4] *Cf.* van Loghem: Centralbl. f. Bakt., I Abt., Orig., 1913, 67:410.

to drying; if a drop of broth culture be dried on a slide, the vibrios are all dead in about two hours. It does not survive long in association with the ordinary saprophytic bacilli of soil and water, and whether it is able to multiply outside the body in impure water is uncertain. Upon the surface of vegetables and fruits kept in a cool, moist place, the vibrios may remain viable for from four to seven days. The slight resistance of the cholera vibrio, especially its sensitiveness to drying, explains the rapid and complete disappearance of cholera in once infected localities, and also the circumstance that the disease is rarely, if ever, air-borne.

The biochemical structure of the vibrios has been studied at some length by Linton and his co-workers.[5] They have described three types of polysaccharide and two types of protein present in the cholera and related vibrios. The poly-
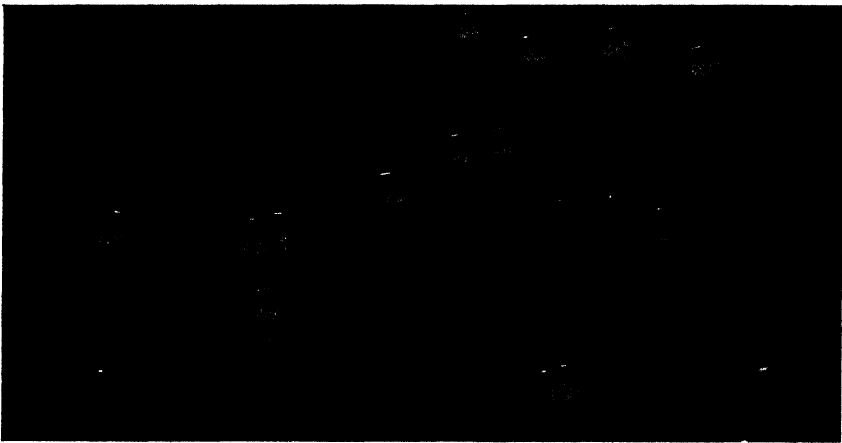
Fig. 87. Colonies of the cholera vibrio on Vedder and Van Dam's medium. The magnification and lighting accentuate the granular structure of the colonies which does not usually appear so pronounced. × 6.

saccharides were differentiated on the basis of their hydrolysis products; that designated type I was made up of galactose and an aldobionic acid (galactose and glucuronic acid), type II of arabinose and aldobionic acid, and type III was a polymer of glucose. The two protein types, both globulins, were differentiated on the basis of racemization, *i.e.*, optical activity of the hydrolyzed protein before and after alkali racemization. These substances occurred in combinations to give six groups of vibrios, the cholera vibrios falling into groups I and II which contained the same protein but polysaccharide types I and II respectively. The significance of these observations is by no means clear. It is evident, however, that the polysaccharide bears no relation to the immunological character of the vibrios for the cholera vibrios are immunologically homogeneous (see below), and it is inconceivable that galactose- and arabinose-containing polysaccharides should show identical immunological specificity. Apparently neither the protein nor polysaccharide fractions are associated with the endotoxin.

[5] Summarized in the review by Linton: Bact. Rev., 1940, 4:261.

Toxin. The evidences of profound toxemia in human infections with the cholera vibrio are strongly suggestive of the formation of toxic substances by this microorganism. It was early shown by Pfeiffer[6] that, while filtrates of young broth cultures were only slightly toxic, vibrios killed with heat or chloroform from young agar cultures were markedly toxic to guinea pigs on intraperitoneal inoculation. The toxicity of cell-free filtrates of old broth cultures led a number of the earlier investigators to the conclusion that a soluble exotoxin is produced. It is now established, however, that no exotoxin is formed and the toxicity of old broth cultures is attributable to dissolution of the vibrios and the liberation of endotoxin.

The endotoxin is heat-stable and it was early found by Metchnikoff to be diffusible through collodion sacs.[7] The isolation of a toxic protein has been reported by some workers[8] and the toxicity has been extracted by the trichloracetic acid method.[9] On the basis of the latter observations it has been assumed that the toxin is a polysaccharide-lipid complex similar to those isolated from typhoid and dysentery bacilli; this conclusion is not, however, supported by unequivocal evidence. More recently it has been found[10] that the cholera toxin is associated, and possibly identical, with a dialyzable phospholipid which may be extracted from the intact vibrios with organic solvents such as alcohol, ether and chloroform.

Though in general the pharmacological activity of the bacterial endotoxins is not characteristic, it has been reported[11] that the intravenous inoculation of experimental animals with toxic preparations produces a diarrhea and pathologic changes in the intestine and kidneys not unlike those observed in human cholera. It has been shown by Burrows, Wagner and Mather[12] that purified toxin markedly increases the permeability of the isolated strip of intestine to fluids. In any case, the cholera toxin seems to have a special affinity for the epithelium and causes a shredding of the epithelium of the intestine and gallbladder in human cholera.

The antigenicity of the cholera toxin has been open to some question for, in general, the antitoxic qualities of cholera antiserum are only feeble at best. Some workers have, however, reported[13] the preparation of effective antitoxin. The purified lipid toxin has been found to be antigenic in that antisera to it

---

[6] Pfeiffer: Ztschr. f. Hyg., 1892, *11*:393; Pfeiffer and Wassermann: *ibid.*, 1893, *14*:46.

[7] For more recent studies see Basu, Chaudhury and Basu: Calcutta Med. Jour., 1940, 36:571; and Banerjee: Jour. Indian Med. Assn., 1942, *11*:95.

[8] Galeotti: Centralbl. f. Bakt., I, Orig., 1912, 67:225; Sanarelli: Ann. Inst. Pasteur., 1920, *34*:370; Hahn and Hirsch: Centralbl. f. Bakt., I, Orig., 1927, *104*:211; *ibid.*, Klin. Woch., 1927, 6:312; *ibid.*, Ztschr. f. Hyg. u. Infektionskr., 1929, *110*:355.

[9] Checcacci: Boll. Inst. Sieroterap. Milanese, 1939, *18*:391; Raynal, Lieou and Feissole: Rev. Immunol., 1939, 5:317; *ibid.*, 1940, 6:132; Damboviceanu and Barber: C. R. Soc. Biol., 1940, *133*:501; Gallut: Ann. Inst. Pasteur, 1943, 69:123.

[10] Burrows: Proc. Soc. Exp. Biol. Med., 1944, 57:306.

[11] Hahn and Hirsch: Klin. Woch., 1928, 7:2483; Gosh: C. R. Soc. Biol., 1933, *112*:1176; Pham: *ibid.*, 1935, *119*:78.

[12] Burrows, Wagner and Mather: Proc. Soc. Exp. Biol. Med., 1944, 57:311.

[13] Pottevin: Bull. Soc. Path. Exot., 1913, 6:409; Horowitz: Ztschr. f. Immunitätsf., 1913, *19*:44; Bail: *ibid.*, 1916, 25:248; *ibid.*, 1917, 26:97; Hahn and Hirsch: Centralbl. f. Bakt., I, Orig., 1927, *104*:211; *ibid.*, Ztschr. f. Hyg. u. Infektionskr., 1929, *110*:355; Andu and van Niekerk: Centralbl. f. Bakt., I, Orig., 1929, *112*:519.

show marked protective qualities as measured by the mouse protection test.[14]

Antigenic Structure. The first demonstration of bacterial agglutination with homologous immune serum by Gruber and Durham[15] was the agglutination of the cholera vibrio and the typhoid bacillus. It was soon apparent that the agglutination reaction was specific and it has been used for the identification of the cholera vibrio since. The antigenic structure of the cholera and related vibrios has, therefore, been of considerable interest in connection with the differentiation of *V. cholerae*.

The existence of serologic types within this group was shown by Japanese workers. Kabeshima[16] demonstrated the existence of two types by cross absorption and designated one "original" or J, and the other "variant" or F. A third serologic type was found by Nobechi[17] which he designated "intermediate" or "middle" since it appeared to have affinities with the other two. The "original" and "variant" types, sometimes called "end types," are now known as Inaba and Ogawa types respectively, and the "middle" type is called Hikojima. All three are equally capable of causing epidemic cholera, but Inaba is most common, practically the only type in India, and Hikojima is least common.

It was shown by Balteanu[18] that vibrios, like other motile bacteria, contain both heat-stable, somatic, or O antigen and heat-labile flagellar or H antigen. Shousha[19] found that the O antigen of the cholera vibrio is specific, while the H antigen is shared with a variety of non-cholera vibrios such as water vibrios and the like. Gardner and Venkatraman[20] have carried this analysis further and found that most vibrios can be differentiated into six groups which they designated by Roman numerals based on the specificity of the O antigen. Group I contains all of the cholera vibrios and some El Tor vibrios. Serological identification of the cholera vibrio, therefore, requires the use of specific O antiserum. Since the El Tor vibrios are hemolytic, the cholera vibrio is defined as a non-hemolytic member of Group I. Gardner and Venkatraman account for the specific agglutination of earlier workers on the basis that O agglutination occurs more rapidly than H agglutination and hence was observed first. A detailed analysis of the O and H antigenic structure of the cholera and related vibrios by Burrows et al.[21] showed that a group-specific O antigen, designated A, is shared by all vibrios of Group I, and that the Japanese types are determined by subsidiary O antigens, B and C, and arbitrarily designated type-specific. It was suggested that the Japanese types be indicated by their antigenic formulae, i.e., Inaba as Type AC, Ogawa as Type AB, and Hikojima as Type ABC. Vibrios containing the group antigen but lacking these type-specific antigens have been found and thus constitute a new type. The H antigens, designated by arabic numerals, and other components of the O antigen are shared by the O Group I vibrios with vibrios of other O groups.

[14] Burrows, Mather, Wagner and McGann: Proc. Soc. Exp. Biol. Med., 1944, 57:308.
[15] Gruber and Durham: Muench. Med. Woch., 1896, 43:285.
[16] Kabeshima: as early as 1912; original articles in Japanese, cited by Nobechi.
[17] Nobechi: Sci. Rept. Govt. Inst. Inf. Dis., Tokyo, 1923, 2:29, 43.
[18] Balteanu: Jour. Path. Bact., 1926, 29:251.
[19] Shousha: Jour. Egypt. Med. Assn., 1931, p. 438.
[20] Gardner and Venkatraman: Jour. Hyg., 1935, 35:262.
[21] Burrows, Mather, McGann and Wagner: Jour. Inf. Dis., 1946, 79:168.

A curious terminology has crept into the cholera literature with respect to agglutination; it is said that a strain which is agglutinated by specific antiserum is "agglutinable" while those vibrios which are not agglutinated by cholera antiserum are called "inagglutinable" despite the fact that they are readily agglutinated by homologous antiserum.

SEROLOGICAL TYPES OF CHOLERA AND PARACHOLERA VIBRIOS

Japanese Type		Immunological Type
Name	Synonyms	
None	None	Type A
Inaba	"J," Japonica 1911, "original," "end type"	Type AC
Ogawa	"F," Formosicana 1911, "variant," "end type"	Type AB
Hikojima	"Middle type"	Type ABC

Variation. The cholera vibrio is well known for its tendency to develop bizarre involution forms. These are found not only in old cultures and cultures grown under somewhat adverse conditions, such as increased salt concentrations, but also are produced by the inclusion of substances such as glycine or alanine in the medium. Such changes in morphology are also associated with the usual type of S-R dissociation which occurs readily under the influence of bacteriophage, lithium chloride, etc., the rough variants showing distinctive colonial character, spontaneous agglutination in salt solution, etc.

The immunological changes associated with the S-R dissociation have been studied in some detail by White.[22] He has found that four groups of rough variants are immunologically distinguishable, and his Group A appears to correspond to the Group I of Gardner and Venkatraman. Further degeneration to the so-called ρ variants results in a loss of specific O antigen. An independent type of variation, rugose-non-rugose, may be induced, and the rugose strains of S, R and ρ variants contain an O antigen that is common to both members of Group A and vibrios of other groups.[23] A somatic protein antigen has been isolated by White[24] which is common to all known variants and shows wide cross-precipitin reactions throughout the vibrio group, although it appears not to be concerned in the agglutination reaction.

Pathogenicity for Man. The causal connection between Asiatic cholera and the microorganism discovered by Koch has been demonstrated by a number of laboratory accidents. One of the first occurred in Koch's laboratory, and other infections resulting from the accidental swallowing of cultures of the cholera vibrio have been noted since. In one instance the swallowing was deliberate; Pettenkofer and Emmerich voluntarily swallowed a small quantity of broth culture of "Koch's vibrio" and as a result developed cholera.

[22] White: Jour. Hyg., 1935, 35:347.
[23] White: Jour. Path. Bact., 1940, 50:160.
[24] *Ibid.*, 1940, 50:165.

Both laboratory cases of cholera and those cases contracted naturally in the course of epidemics are marked by great differences in the susceptibility of different individuals. This is probably a consequence in part of innate individual differences in natural resistance, and in part to predisposing factors. Fatigue, the excessive use of alcohol, and various factors leading to mild, non-specific gastro-intestinal derangements predispose in a marked degree to attacks of cholera.

The incubation period is short; it is usually given as three to five days but may occasionally be as short as twenty-four hours. When the vibrios have entered the small intestine and established themselves they multiply rapidly and, with autolysis and dissolution of the cells, endotoxin is liberated and symptoms appear. There are two clinical stages, the first that of *vomiting* and *profuse diarrhea* with the characteristic ricewater stools containing flakes of mucus, shed epithelial cells and enormous numbers of the vibrios. With the tremendous loss of fluid dehydration and hypochloremia become marked, and collapse, the *algid stage*, ensues with circulatory failure, subnormal temperature and anuria. Death may occur in either the first or second stage. The case fatality rate is over 50 per cent in untreated cases and about 30 per cent in treated cases.

At autopsy the small intestine shows marked destruction of the epithelial lining and a characteristic subepithelial edema. It is generally said that ulceration does not occur in cholera, but Goodpasture[25] has shown that desquamation of the epithelium is followed by ulceration if the patient survives long enough. Other changes are not characteristic and include a prominence of lymphoid tissue and cloudy swelling of the kidneys.[26]

Cholera in man appears to be largely, if not entirely, a toxemia, for the infection is confined to the lumen of the intestine and vibrios are found in the organs only rarely. It is not altogether clear whether toxin is absorbed. In the opinion of many the profound dehydration and hypochloremia and consequent impairment of renal function are sufficient to cause the clinical symptoms and pathology.

Carriers. On recovery the vibrios disappear rapidly and are discharged for only a short time. In the series of 200 cases studied by Ying[27] 98 per cent were negative by the end of the second week, only a few giving positive cultures as late as the third and fourth weeks. There appears to be no chronic carrier state established in cholera. The statements in the literature regarding chronic carriers refer to persons who discharge "agglutinable" vibrios of the El Tor type; a chronic carrier of typical virulent cholera vibrios has never been observed.[28] It is probable that casual carriers occur during epidemics but in no instance has this been demonstrated bacteriologically. While the convalescent carrier, and possibly the casual carrier, may play some part in the spread of the disease, the case, especially in the incubation period, is probably by far the most important source of infection.

[25] Goodpasture: Philippine Jour. Sci., 1923, 22:413.

[26] The pathology is summarized briefly by Banerjee: Jour. Indian Med. Assn., 1939, 8:39.

[27] Ying: Chinese Med. Jour., 1940, 58:595.

[28] The carrier question is discussed by Couvy: Bull. Office Internat. d'Hyg. Publique, 1933, 25:1149.

Bacteriological Diagnosis of Asiatic Cholera. As indicated above, the vibrios are present in the ricewater stools in very large numbers and can usually be isolated from fresh specimens without difficulty. At times they are present in sufficient numbers that they can be found in stained smears, preferably made from a flake of mucus. Both enrichment and direct streaking of agar media are used for cultures. For enrichment a few drops of the stool are added to a tube of alkaline (pH 8.0 to 9.0) peptone water. The vibrios grow much more rapidly than the other intestinal bacteria and after six to eight hours' incubation form a thin film of growth on the surface of the medium which can be smeared and stained, and streaked on agar.

A number of agar media have been used for isolation of the cholera vibrio, including starch-phenolphthalein agar and the alkaline blood medium of Dieudonné. The latter has been the most generally used. In its preparation an excess òf alkali is added and the poured plate must be ripened, *i.e.*, left partly open in the incubator for twenty-four hours to allow the evolved ammonia to escape. With storage the pH continues to drop and the medium becomes less selective and unsatisfactory. The alkaline hemoglobin medium of Vedder and Van Dam[29] is buffered with glycine, requires no ripening, and the pH remains constant at 9.5 with storage. It possesses all the advantages and none of the disadvantages of Dieudonné's medium. It is sometimes implied that these media with a pH at the upper limit of tolerance of the cholera vibrio are so highly selective that few if any other intestinal bacteria will grow on them. This is not true, for micrococci may be found in abundance on such plates; the writer has observed counts of 1000 million bacteria per gram of normal guinea pig feces on both Dieudonné's and Vedder and Van Dam's media. However, colonies of the cholera vibrio are readily distinguished and may be picked and identified by slide agglutination with monospecific O antiserum. With clinically typical cholera in an epidemic this is sufficient identification. A negative Greig test, fermentation of sucrose and mannose and failure to ferment arabinose, and positive indol and nitrite (cholera-red) reactions further substantiate the identification.

Epidemiology. As indicated earlier, the great endemic focus of Asiatic cholera is in India, especially in the delta of the Ganges River. Recent evidence has indicated that there is also an endemic area in China, in the valley of the Yuan River which flows into the Yangtse River through Tun Ting Lake.[30] Whether this is recently established or recently discovered is not known. In any case, the disease spreads out in epidemic form from these foci each year, occurring regularly throughout India, in the Yangtse Valley and along the China coast. The entire Far East is affected at one time or another, especially China and French Indo-China, and the disease appears from time to time in Manchuria and Japan. The last general outbreak in the Philippine Islands was in 1934; a few cases appeared in 1935 and 1936. The geographic distribution differs greatly from year to year and that indicated in Fig. 88 is only an approximation.

Infection spreads to the west especially via the pilgrimages; pilgrims going from the delta of the Ganges to Arabia and Mecca transmit it to pilgrims from

[29] Vedder and Van Dam: Nederl. Tijdschr. v. Hyg. Microbiol. en Serol., 1932, 7:197.
[30] Robertson and Pollitzer: Trans. Roy. Soc. Trop. Med. Hyg., 1939, 33:213.

Egypt and Algiers. The quarantine stations at El Tor and Basra (see map) serve to keep the disease out of Europe in normal times though it seeps through into Iran and Iraq with some frequency. There was cholera in Europe during the Balkan War and the First World War and the disease occurred in epidemic form in Eastern Europe in 1921 and 1922. There has been no cholera in the United States proper since 1911.

As in the other enteric infections, the connecting link in the dissemination of cholera is between infected feces and the mouths of susceptible persons. In

APPROXIMATE DISTRIBUTION
OF ASIATIC CHOLERA

ENDEMIC CENTERS

FREQUENTLY EPIDEMIC

OCCASIONALLY EPIDEMIC

⊕ SPORADIC
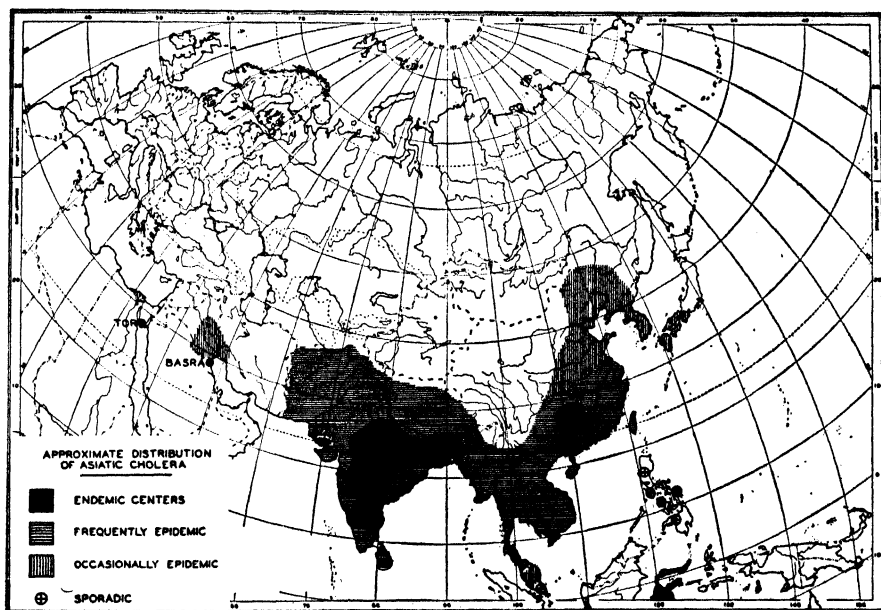
Fig. 88. The approximate distribution of Asiatic cholera. The precise distribution cannot be shown because of great variability from time to time. This represents an approximate distribution during the decade 1930–1940. Based in part on map prepared by Army Medical Intelligence, 1943, epidemiological reports of the League of Nations, and various authors. (Based on Goode *Base Map* No. 205. By permission of the University of Chicago Press.)

consequence, the disease is frequently water-borne and may be transmitted by any food ordinarily consumed in the raw state. The quantitative importance of contact infection is not known. Cholera differs from the other enteric diseases, however, in the highly explosive character of the outbreaks, which is attributable to the short incubation period, the high case fatality rate, and its rapid and permanent disappearance when the outbreak has subsided. The last is due in part, perhaps, to the inability of the vibrio to survive long apart from the host, but more important is the limited duration of the convalescent carrier state and the absence of chronic carriers. In a sense cholera is one of the easiest to control of the highly contagious diseases for it cannot spread when sanitary facilities, *i.e.*, sewage disposal, water supply, etc., are in efficient operation. A striking instance of this was reported in the Balkan War in 1913 in which infection was widespread in the Bulgarian Army about Sofia, but cases in the

capital were largely imported and the disease showed little tendency to spread there; Sofia was efficiently sewered and had an excellent water supply.[31] Contrariwise, the famous Broad Street Pump epidemic in London in 1854 was made possible by the common use of a continuously infected well,[32] and the Hamburg epidemic of 1892 arose from infection of the river Elbe. The Hamburg water supply was raw river water, but the neighboring city of Altona purified the water by sand filtration and remained free of the disease.[33]

Immunity. Antibodies, agglutinins and bacteriolysins, are produced by infection with the cholera vibrio but have no diagnostic utility because of late and irregular appearance. It is usually said that recovery from an attack of cholera confers an immunity to subsequent infection, but neither the quality of this immunity nor its duration is definitely known. It seems probable that
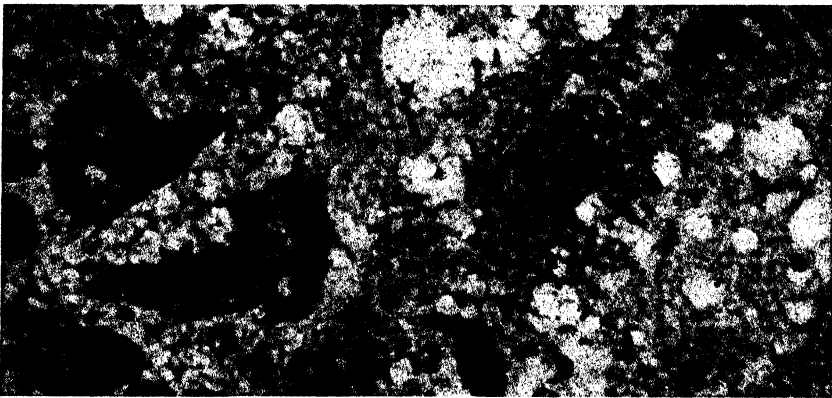
Fig. 89. *Vibrio cholerae* in peritoneal exudate of a guinea pig. Note the swollen and aberrant forms. Gram stain; × 1250.

the immunity is not of a high order and that it does not persist more than a year or two at the most.

Active immunization of man with vaccines has been carried out for many years. The first vaccine was that of Haffkine (1893) who used a living attenuated culture in Spain and India.[34] A heat-killed vaccine was used by Kolle, and was reported to give good results in Japan. Sensitized vaccines, *i.e.*, vibrios treated with antiserum, have been investigated also.[35] The cholera vaccine used at the present time in this country and in India is a saline suspension of killed vibrios, half the Inaba variety and half the Ogawa variety. Cholera vaccines are usually heavier than the vaccines prepared from other enteric bacilli, and contain 8 to 10 billion vibrios per milliliter. Usually two doses of 1 ml. each are given a week apart, and a booster dose of 1 ml. every six months.

It is not clear how effective active immunization is. Much of the reported evidence is open to criticism and not convincing. The difficulties of carrying out

[31] Eckert: Berlin. Klin. Woch., 1913, 50:2326.

[32] *Snow on Cholera.* The Commonwealth Fund, New York. 1936.

[33] Koch: Ztschr. f. Hyg., 1893, 14:393.

[34] A history of this work is given by Haffkine: *Protective Inoculation Against Cholera.* W. Thacker & Co., London. 1913.

[35] *Cf.* Takenouchi: Jour. Inf. Dis., 1920, 26:441.

an adequately controlled experiment in man are, of course, very great indeed. The earlier work was examined by Greenwood and Yule[36] who found it unconvincing on the whole, but there was a slight presumption in favor of inoculation. Most subsequent reports have been favorable with a few definitely unfavorable. An extensive and carefully controlled field study was carried out in Madras in 1941–42 and analysis of the results indicated a definite protective effect of prophylactic inoculation, the immunes being twelve to fourteen times less likely to get the disease.[37] This is the best evidence yet presented for the efficacy of cholera vaccination. It is generally agreed that cholera is as severe in the inoculated as in the uninoculated and the therapeutic use of antisera is apparently without effect. It may be noted that the problem of active immunization to cholera is very similar to that of bacillary dysentery (p. 476).

Pathogenicity for Lower Animals. Under natural conditions domestic animals and the animals used in the laboratory never contract cholera. It is possible to produce enteric infection with the cholera vibrio in the guinea pig by oral administration (by stomach tube) of very large numbers of the vibrios either suspended in alkaline buffer or following oral administration of bicarbonate, provided that intestinal motility has been reduced by prior administration of tincture of opium. A true infection is produced with active multiplication of vibrios in the intestine, but without generalized invasion of the body, and with their excretion in the feces which persists for ten to fourteen days and occasionally as long as three weeks in non-fatal infection.[38] The suckling rabbit may be similarly infected and is considerably more susceptible than the guinea pig, but after weaning cannot be infected. It is not clear how closely related these experimental infections are to human cholera.

The guinea pig is susceptible to intraperitoneal inoculation of living vibrios and "intraperitoneal cholera" has been extensively studied by the earlier workers. Recently Griffitts[39] has shown that the virulence of the cholera vibrio for mice by intraperitoneal inoculation may be markedly enhanced by suspension in 5 per cent mucin; as few as 50 living vibrios may kill by this method. This will undoubtedly be of considerable utility in assay of virulence and active and passive immunity, but would appear to be quite unrelated to the enteric infection of man. The developing chick embryo may also be infected with the cholera vibrio, and is protected by the inoculation of immune serum.[40]

Active or passive immunization of experimental animals to parenteral inoculation of the cholera vibrio is highly successful, and the usual immunization procedures yield high titer antisera. The immunity so conferred is apparently almost wholly an antibacterial immunity for little antitoxic activity may be detected. It was shown by Pfeiffer[41] that lysis of the vibrios by immune serum occurs readily. The *Pfeiffer phenomenon* may be demonstrated in either actively or passively immunized guinea pigs by intraperitoneal inoculation

[36] Greenwood and Yule: Proc. Roy. Soc. Med., Sec. Epidemiol. and State Med., 1915, 8:113.

[37] Lancet: 1946, ii:134; Adiseshan, Pandit and Venkatraman: Indian Jour. Med. Res., 1947, 35:131.

[38] Burrows, Elliott and Havens: Jour. Inf. Dis., 1947, 81:261.

[39] Griffitts: Pub. Health Repts., 1942, 57:707.

[40] Wilson: Jour. Exp. Med., 1946, 84:305.

[41] Pfeiffer: Ztschr. f. Hyg., 1894, 18:1; ibid., 1895, 20:198.

of living vibrios; if peritoneal fluid is withdrawn from time to time and examined, it will be found that the vibrios lose their motility, become swollen and misshapen and then distintegrate. The phenomenon may be produced *in vitro* with potent antiserum and complement.

PARACHOLERA VIBRIOS AND NON-CHOLERA VIBRIOS

For the most part as an adjunct to the study of the cholera vibrio, a considerable number of vibrios have been isolated from water and from the feces of individuals suffering from mild diarrheal disease, so-called cholera nostra. Some of these have been given place names such as *Vibrio danubicus, Vibrio ghinda, Vibrio massauah,* etc. A phosphorescent vibrio, *Vibrio phosphorescens,* has been isolated from water, and *Vibrio proteus* from human feces. All of these differ immunologically from the cholera vibrio, *i.e.,* belong to O Groups other than I, and have faded from general interest. Some may possibly have feeble pathogenic properties but in tropical and subtropical regions where diarrheal disease is common (and of varied etiology) and great numbers of vibrios are present in water used for drinking, it is not surprising that they are occasionally found in fecal specimens though unrelated to a disease condition. In general, the group of vibrios is very poorly known from the biological point of view.

The El Tor Vibrios. The best known of the so-called paracholera vibrios are those which were first isolated at the Tor quarantine station in 1906 from pilgrims suffering from diarrheal disease. A considerable number of strains was isolated at Tor in 1930 and 1931 by Doorenbos, and apparently these vibrios can be isolated with some frequency there. Very many, though not all, of the El Tor strains are "agglutinable," that is to say, agglutinate with anticholera sera, and have been shown to belong to O Group I by Gardner and Venkatraman.[20] These, like the cholera vibrio, contain both group- and type-specific O antigens, giving rise to El Tor vibrios of the Inaba type or Type AC, etc. The agglutinable strains differ from the cholera vibrio only in that they are actively hemolytic as noted earlier; a number of physiological tests, such as the Voges-Proskauer reaction, have been said to differentiate the two but none has proved to be reliable.

The relationship of these vibrios to Asiatic cholera has been a matter of considerable interest; aside from general considerations, their presence raises a specific practical question at quarantine stations. Doorenbos[42] is strongly of the opinion that two types of cholera may be distinguished, the epidemic form caused by the classic cholera vibrio, and the endemic form caused by atypical variants of *V. cholerae* such as the El Tor vibrio; inherent in this is the assumption that the El Tor vibrios may become typical *V. cholerae.* This view is not generally accepted and it is believed that the El Tor vibrio, sometimes given the specific name *Vibrio El Tor,* has some pathogenic powers and may be causally associated with diarrheal disease, but neither the vibrio nor the disease is related to *V. cholerae* and Asiatic cholera in the sense of Doorenbos.

The Celebes Vibrio. The question of the pathogenicity of the hemolytic agglutinable vibrios was raised again by the occurrence of an epidemic, apparently of cholera, in Celebes in 1937 and 1938 which was reported by de

[42] Doorenbos: Rev. d' Hyg. et de Med. Preventive, 1936, 58:595, 675, 736; *ibid.,* 1937, 59:22, 105; Jour. Egypt. Med. Assn., 1938, 21:279.

Moor.[43] About 400 strains of the vibrio were isolated and all proved to be hemolytic and agglutinable with cholera antiserum; van Loghem[44] found representative strains to be indistinguishable from the El Tor vibrio. With the outbreak of war in Europe and the Far East these strains were no longer available and do not seem to have been studied further. This epidemic, in which many cases were clinically indistinguishable from cholera though some were mild, is convincing evidence of a high degree of pathogenicity of at least some strains of the hemolytic agglutinable vibrios.

Other Pathogenic Vibrios. A number of other vibrios are known which produce disease in animals but which are apparently not pathogenic for man. *Vibrio metchnikovii* was isolated in 1888 from fowls suffering from an epidemic disease resembling fowl cholera. It closely resembles the cholera vibrio morphologically and physiologically and is highly pathogenic for guinea pigs and pigeons while the cholera vibrio is not pathogenic for the latter. It differs from *V. cholerae* immunologically and is neither agglutinated nor lysed by anticholera serum. An infectious abortion of sheep caused by *Vibrio fetus* was described by McFadyean and Stockman[45] in England and also occurs in this country but is not prevalent. The vibrio is relatively difficult to cultivate and is biochemically inactive, fermenting none of the sugars. It is not pathogenic for guinea pigs. An epidemic disease of carp and other fish caused by a vibrio designated *Vibrio piscium* has been described by David.[46] The vibrio resembled the cholera vibrio morphologically, and was immunologically related to it. A vibrio to which no formal name was assigned has been described by Doyle[47] as associated with swine dysentery. This vibrio was very difficult to cultivate and its relationship to the other vibrios is not as yet clear.

[43] de Moor: Bull. Office Internat. d'Hyg. Publique, 1938, 30:1510; see also de Vogel: *ibid.*, 1940, 32:556.

[44] van Loghem: Bull. Office Internat. d'Hyg. Publique, 1938, 30:1520.

[45] McFadyean and Stockman: Report of the Departmental Committee appointed by the Department of Agriculture and Fisheries to inquire into epizootic abortion. 1909. Part 1, p. 15. For more recent studies see Plastridge *et al.*: Amer. Jour. Vet. Res., 1947, 8:178; and Rhoades: Cornell Vet., 1947, 37:8.

[46] David: Centralbl. f. Bakt., I Abt., Orig., 1927, 102:46.

[47] Doyle: Amer. Jour. Vet. Res., 1944, 5:3.

BRUCELLA[1]

Undulant Fever, Contagious Abortion of Cattle

In 1887 Bruce, while investigating the human disease known as Malta fever, Mediterranean fever or undulant fever, discovered a microorganism in the spleen of fatal cases of the disease which he designated *Micrococcus melitensis.* A disease of goats transmissible to man, this affection not only is common on the island of Malta, where British garrisons have been often seriously affected, but occurs also on neighboring islands and on the shores of the Mediterranean Sea, and has been occasionally reported from India, South Africa, the Philippines and the West Indies. It was first brought to attention in the United States about 1911.

In 1897 Bang, in Denmark, isolated a microorganism responsible for a contagious abortion in cattle, an affection now commonly known as Bang's disease, which he termed *Bacillus abortus.* The isolation and cultivation of this bacterium in the United States were first recorded by MacNeal and Kerr in 1910.

These two diseases, one primarily of goats and secondarily of man, and the other, one of cattle, were long studied quite independently, and apparently no connection between the two was recognized prior to the work of Evans[2] in 1918. This worker demonstrated the remarkably close morphological, cultural and serological relationship existing between these bacteria which are now recognized as being intimately related to one another.

In 1914 Traum isolated from fetuses prematurely expelled from sows a bacterium which is now known to be closely related to the bacterium of Bang's disease and that of undulant fever. Regarded as three species, these bacteria have been given the generic name of Brucella and are designated as *Brucella melitensis, Brucella abortus* and *Brucella suis.* Infection with these bacteria is often termed "brucellosis."

Morphology and Staining. The Brucella are small coccoid or short bacillary forms varying from 0.4 to 3.0 μ in length and from 0.4 to 0.8 μ in breadth. Some variability is noted, and both coccoid and bacillary forms may appear intermingled. There is a greater tendency to the coccobacillary form in *Br. melitensis* than in *Br. suis,* with *Br. abortus* intermediate be-

---

[1] For a detailed discussion of these bacteria see Huddleson: *Brucellosis in Man and Animals.* 2nd ed. The Commonwealth Fund, New York. 1939.

[2] See the general discussion by Evans: Amer. Jour. Pub. Health, 1947, 37:139.

tween the two, but no distinction can be made on a morphological basis. The microorganisms usually occur singly or in pairs, and in cultures short chains may be found. The smooth forms are encapsulated but spores are not formed, and these bacteria are non-motile.

On semisolid media the colonies are small, circular, convex, amorphous, smooth, glistening and translucent. No pigment is formed, but the growth of *Br. melitensis* becomes brown in older cultures and the browning extends down into the medium. This browning is shown by some strains of *Br. abortus* also.

Brucella may be stained by the usual aniline dyes, but there is a tendency toward irregular staining and, in some cases, bipolar staining. They are gram-negative.
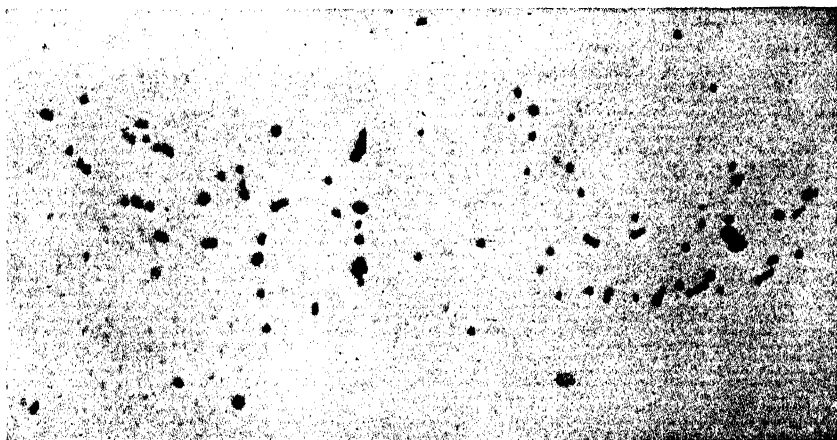
Fig. 90. *Brucella melitensis*, pure culture. Note the coccobacillary appearance. Fuchsin; × 1050.

Physiology. The nutritive requirements of these bacteria are relatively complex and best growth is obtained on enriched media such as liver infusion broth or agar. Kerby and Calder[3] have reported that a milk-tryptose-crystal violet medium is superior to liver infusion broth or tryptose broth in primary culture. Brucella have been cultivated on amino acid synthetic media; some strains require nicotinamide, thiamine and pantothenic acid while others require biotin also. A number of chemically defined or synthetic media have been prepared which support the growth of Brucella, one of the simplest of which is that of Gerhart and Wilson[4] containing lactate, glycerol, asparagin (or glutamic acid or histidine), thiamine, nicotinic acid, pantothenic acid and biotin together with inorganic salts. No growth occurs at 6° C. or 45° C. and the optimum temperature is 37° C. Neither acid nor gas is produced from carbohydrate media, although it may be shown that glucose is utilized to a small extent and its inclusion generally favors growth. Nitrates are re-

[3] Kerby and Calder: Jour. Bact., 1940, 40:637.
[4] Gerhardt and Wilson: Jour. Bact., 1948, 56:17; see also the studies of McCullough et al.: *ibid.*, 1947, 53:5.

duced, and growth in milk is accompanied only by a slowly increasing alkalinity. Gelatin is not liquefied and indol is not formed. The optimum pH is 6.6 to 6.8.

Hydrogen Sulfide. All three species produce hydrogen sulfide but differ in that *Br. suis* produces it in abundance, *Br. abortus* to a lesser extent and *Br. melitensis* to only a slight degree. It may be noted that ammonia is produced to a greater extent by *Br. melitensis* than by the other two species.

Carbon Dioxide. These bacteria are aerobic, and *Br. melitensis* and *Br. suis* may be grown on primary isolation under the usual aerobic conditions. *Br. abortus,* however, requires incubation in an atmosphere containing 10 per cent carbon dioxide on primary isolation. Subsequent transfers from the primary growth must be incubated in 10 per cent carbon dioxide, but after a number of transfers *Br. abortus* adapts itself to ordinary aerobic growth.

Fig. 91. *Brucella melitensis.* Pure culture on liver infusion agar. × 4.

Dyes. The Brucella species differ from one another in their susceptibility to the bacteriostatic effect of dyes, and their ability to grow on liver infusion agar containing thionine or basic fuchsin is a characteristic of considerable practical value in their differentiation. *Br. melitensis* and *Br. abortus* will grow in the presence of basic fuchsin in a dilution of 1 : 25,000, but *Br. suis* is completely inhibited. *Br. melitensis* and *Br. suis* will grow in the presence of thionine in a dilution of 1 : 50,000, while *Br. abortus* will not. These and other cultural differences are summarized in the accompanying table.

The Brucella show the usual susceptibility to heat and disinfectants. A point of some practical importance is the rapid death of these bacteria at pasteurizing temperatures; both *Br. abortus* and *Br. suis* are killed in three minutes at 143° to 145° F. They persist in soil, water and dust for one to two months but disappear within ten days in milk, presumably in part as a consequence of the presence of acids formed by other bacteria. In this connection it is of interest that these bacteria are able to survive two hours or more in milk mixed with gastric juice. ·

Antigenic Structure. Each of the three Brucella species contains two antigens, designated as *A* and *M*. *Br. melitensis* contains a relatively large amount of *M* and a small amount of *A*, while both *Br. abortus* and *Br. suis* contain large amounts of *A* and small amounts of *M*.[5] The ratio of *A* to *M* is said to be about 20 : 1 in the case of *Br. abortus* and 1 : 20 in the case of *Br. melitensis*. It is possible, then, to differentiate *Br. melitensis* from *Br. abortus* and *Br. suis* by serological methods, *i.e.*, agglutination, but *Br. abortus* and *Br. suis* cannot be differentiated from one another in this way. In practice monospecific sera, *i.e.*, sera adsorbed to remove the small amount of antibody common to the other immunological type, should be used. These antigens are both heat-stable, and their chemical nature is as yet unknown.[6]

CULTURAL DIFFERENCES OF BRUCELLA SPECIES

	CO₂ Required for Growth	H₂S Production	Growth in Presence Of	
			Basic Fuchsin 1:25,000	Thionine 1:50,000
Br. melitensis............	−	±	+	+
Br. abortus..............	+	+	+	−
Br. suis................	−	++	−	+

Variation. The Brucella species dissociate relatively easily to give rise to the rough form. The environmental factors affecting the dissociative process have been studied in detail by Braun.[7] The S-R transformation is accompanied by a change to a rough colonial type, a loss of virulence and alteration in immunological specificity. The last begins to take place before morphological changes are apparent and has been a source of considerable difficulty in the serological typing of these bacteria; the altered immunological types have been termed *paramelitensis* or *para-abortus* strains. It is, therefore, essential to use only smooth cultures in the serological differentiation of these bacteria.

A number of tests have been devised for the detection of antigenic variants. The rough forms are, of course, spontaneously agglutinated in saline. Slightly rough strains may be detected by their agglutination upon boiling in saline for two hours (thermo-agglutination test) or by incubating saline suspensions of the bacteria with basic fuchsin or trypaflavine at 37° C. for two hours; the rough form agglutinates in the presence of the dye while the smooth form remains evenly suspended. According to Huddleson,[8] however, none of these gives consistent results, and he has proposed an

[5] Wilson and Miles: Brit. Jour. Exp. Path., 1932, *13*:1.
[6] *Cf.* the investigations of Miles and Pirie: Brit. Jour. Exp. Path., 1939, 20:83, 109, 278; Biochem. Jour., 1939, 33:1709, 1716.
[7] Braun: Jour. Bact., 1946, *51*:327.
[8] *Cf.* Munger and Huddleson: Jour. Bact., 1938, 35:255.

opsonocytophagic test in which bacterial suspension is mixed with citrated normal guinea pig or human blood, incubated for thirty minutes, smeared and stained. Smooth strains show only a slight degree of phagocytosis while the rough bacteria are phagocytosed to a considerable extent.

Pathogenicity for Lower Animals. Brucellosis is primarily a disease of domestic animals and is only secondarily communicated to man; the chief animal reservoirs are goats, cattle and swine. It is of some interest that host specialization of the parasites has taken place, giving rise to the three species or, as some prefer to regard them, varieties of Brucella.

Goats. Goats may be artificially infected with *Br. melitensis* by almost any route, and it is probable that under natural conditions the vaginal discharge at the time of aborting and shortly thereafter plays an important part in the dissemination of the infection. Agglutinins appear in the blood of artificially inoculated pregnant goats by the third or fourth day, and the titer rises rapidly to perhaps 1:1000 within forty-eight hours and reaches a peak of 1:2000 or thereabouts by the twelfth day of infection. Just before the peak in agglutinin titer, a bacteremia is initiated which persists for perhaps one month. This acute generalized infection becomes localized during the second month after the termination of the pregnancy during which the animal was infected. In most cases the bacteria do not persist in the udder and uterus after the fifth month following termination of pregnancy. A second pregnancy does not, as a rule, cause an exacerbation of the disease, but in some cases the infection may remain localized in the area of the genital tract for several years.

The most obvious clinical symptom of infection is abortion, although this need not occur. Pyrexia is apparent within forty-eight hours of the generalized infection, and there is a slight diarrhea. The placenta is not retained, but a copious vaginal discharge is frequently observed for two or three weeks after kidding. In lactating goats the milk may be physically altered and appear in extreme cases as a clear fluid containing suspended clots.

Immature goats are highly resistant to the infection, and kids born of infected dams may not be infected and commonly do not become so in spite of the ingestion of enormous numbers of Brucella in the milk. Nonpregnant mature goats are also resistant to infection and respond to artificial inoculation with only a low and transient agglutinin titer in the blood serum.

Brucellosis in sheep is similar to that in goats.

Cattle. Brucellosis in cattle is most commonly an infection with *Br. abortus* although both *Br. melitensis* and *Br. suis* have also been found. The microorganism may gain entrance by a variety of routes, including direct inoculation into the vagina, by way of the conjunctiva, through the unbroken skin or via the alimentary tract. The primary symptom of the disease is abortion of the fetus by pregnant cattle.[9] The time elapsing between initial infection and abortion varies from three weeks to four months, and the period of gestation at which abortion may take place varies from

[9] Abortion may, of course, result from other infections. See the discussion by Gilman: Cornell Vet., 1939, 29:153.

two to nine months. Cattle do not abort, however, unless infected during pregnancy and even then not all abort—perhaps 30 per cent—or the cattle may become sterile. Subsequent pregnancies may proceed normally in spite of persistence of the infection; second abortions are not common, and third abortions are rare.

The bacilli may be found in the blood in perhaps 10 per cent of the cases and are very likely consistently present during the acute infection. Early in the infection the bacteria are found in the lymph glands about the head and intestines, by the end of the first month they are found all through the body, and by the end of the third month have localized in the mammary glands and are found only in the udder. The invasion of the udder results in an acute or chronic inflammation with lesions in the alveoli and interalveolar connective tissue and, when the lymph glands are involved, a chronic lymphadenitis. Chronic infection of the udder may persist indefinitely without significant differences in the quality of the milk and bacilli may be excreted over a long period of time, perhaps for life. The uterus, on the other hand, frees itself of the bacteria relatively soon and the vaginal discharges do not contain the bacilli for an extended period.

Animals infected during pregnancy show an agglutinin titer ranging from 1:200 to 1:1000 which falls slowly over a period of six months or so. Cattle that continue to excrete bacilli in the milk generally show persistent agglutinin titers of 1:200 or more although a titer of 1:50 has diagnostic significance. Agglutinins are also present in the milk and may be demonstrated in the whey after clotting with rennin. Infected animals become sensitized to the bacillary substance, and a skin reaction may be elicited by the intradermal injection of a preparation of Brucella protein designated as *abortin* or *brucellergen*. As in the case of the young goats, calves are relatively resistant to the infection.

Swine.[10] Brucellosis of swine seems to be always due to infection with *Br. suis,* though these animals may be artificially infected with *Br. abortus.* In contrast with cattle, the males are commonly infected and abortion in infected females is less frequent than in cows; about 50 per cent of swine abortions are due to unknown causes, not brucellosis. The clinical symptoms may be mild or lacking, and in a number of instances there has been no outward evidence of the disease in an infected herd but the proportion of swine infected is as high as 20 per cent in some localities. Under natural conditions infection probably takes place via the alimentary tract. The bacilli are eliminated with aborted fetuses and vaginal discharge, urine, semen and milk.

Other Animals. A number of other animals have been found to be naturally infected with Brucella. There is some evidence that the disease of horses known as fistula of the withers or poll evil is a Brucella infection; both *Br. abortus* and *Br. suis* have been isolated from cases of the disease. Stone[11] found that 9.5 per cent of horses tested in New York City gave positive serological reactions. Brucella infections of fowl have been reported; in one instance *Br. suis* was isolated from several naturally infected

[10] See the general discussion by Hutchings: Mich. State Coll. Vet., 1944, 4:68, 69, 86.
[11] Stone: Jour. Amer. Vet. Med. Assn., 1941, 99:118.

birds, but the disease is probably not common. Dogs may also be infected naturally; *Br. suis* and *Br. abortus* have been isolated. Wild rats may be artificially infected, and *Br. abortus* has been isolated from a naturally infected rat. Natural infection of rabbits with *Br. melitensis* has also been reported.[12] Of the usual laboratory animals guinea pigs are readily infected and are most often used for experimental purposes. Rabbits, mice and other animals may also be infected. A disease resembling undulant fever in man has been produced in rhesus monkeys.

Pathogenicity for Man. Man is susceptible to infection with these three species of Brucella, but infections with *Br. melitensis* and *Br. suis* are usually more severe than those with *Br. abortus*. The incubation period of undulant fever in man is highly variable and relatively long; it may range from one week to not less than four months. The case fatality is low—2 to 3 per cent. It may have varied clinical manifestations and 5 types are recognized: (1) the intermittent type with shifting articular rheumatism, weakness, night sweats and a temperature near normal in the morning but rising to 101° to 104° F. in the evening, in which the patient remains in bed in the latter part of the day; (2) the ambulatory type with much the same symptoms but to a mild degree; (3) the undulant type, generally melitensis infections, characterized by step-like increases in the temperature from day to day to a maximum and, after a time, gradual decrease in temperature and possibly successive repetitions of this sequence of events; (4) the malignant type, almost always melitensis infections, in which the temperature is high and sustained with an extreme hyperpyrexia before death; and (5) an atypical chronic type which may take the form of muscular stiffness, gastric disturbances and various neurological symptoms. In general, undulant fever is a disease of relatively long duration, one to four months, and relapses during convalescence are not infrequent. In a chronic form undulant fever may be difficult to diagnose.

Brucellosis in man is a generalized infection as a rule, while in lower animals it is a localized infection, particularly in cattle. The bacilli may be isolated from the blood stream in man and the development of agglutinins is a diagnostic aid. Man also becomes sensitized to the cell substance of the bacteria, a hypersensitivity that is sometimes manifested as skin eruptions which may be macular or resemble the rose spots of typhoid fever. Localization may occur, however, and meningitis and meningo-encephalitis are probably not so rare as has been supposed, while in some instances orchitis, cholecystitis, endocarditis and other local manifestations have been reported. Pulmonary lesions with infiltration of the hiliar glands or lung tissue proper are occasionally observed. Pulmonary infection has led many investigators to suspect infection by inhalation, and Elberg and Henderson[18] have shown that the guinea pig may be experimentally infected by an intake of about 36 microorganisms inhaled as an aerosol. Brucella infection may be associated with abortion and mastitis in the human female in rare instances.[14]

[12] For a discussion of brucellosis in wildlife see the review by Katz: Jour. Amer. Vet. Med. Assn., 1941, *99*:24.

[18] Elberg and Henderson: Jour. Inf. Dis., 1948, *82*:302.

[14] The clinical aspects of human brucellosis are discussed in detail by Harris: *Brucellosis*. Paul B. Hoeber, New York. 1941; *ibid.*: Jour. Amer. Med. Assn., 1946, *131*:1485.

Epidemiology Brucellosis in man is probably always acquired from infected domestic animals; man-to-man transmission is a possibility but rarely, if ever, occurs. The commonest modes of infection in the United States are, first, the use of raw milk from infected cattle and, second, direct contact with the flesh of infected animals, both cattle and swine. As indicated above, animals may be readily infected via the alimentary tract, and it is not unreasonable to suppose that man is infected in this way also. The discharge of *Br. abortus* in the milk of infected cattle, then, provides the opportunity for infection when the milk is ingested in the raw state and in many instances undulant fever is acquired in this way. *Br. abortus* has been found in certified milk in a number of localities.[15] The pasteurization of milk, of course, provides adequate protection from this source of infection.

The penetration of the unbroken skin by Brucella has been pointed out earlier. Man may be infected by the handling of the tissues of diseased animals or by close contact with other infectious material; presumably the bacilli enter through minute abrasions in the skin, or possibly through the intact skin. Employees of slaughterhouses, veterinarians, sausage-makers and butchers are, of course, particularly exposed to infection by this means and, in fact, the incidence of brucellosis in this group is disproportionately high. It is probable that most infections with *Br. suis* are acquired in this manner, although in some instances cattle are infected with this species and man acquires a *suis* infection via raw cow's milk.

Laboratory infections with Brucella are very common and even the most skilled workers have acquired undulant fever through working with these bacteria. Meyer and Eddie[16] have reviewed 74 cases up to 1940 of which 44 occurred in competent bacteriologists. These infections are, in all probability, a consequence of handling infectious material and penetration of the skin by the microorganisms.

Undulant fever may also be acquired by drinking raw goat's milk, but infections with *Br. melitensis* from these animals are thought to be relatively infrequent in the United States. Evans,[17] however, has found a number of cases in North Carolina, Kansas and Texas, and it is known to occur in the Southwest in general where goat's milk is consumed.

It has been shown experimentally that brucellosis may be transmitted by mosquitoes and biting flies, but at present there is no indication that this mode of transmission is of any significance in nature. Water is apparently not a vehicle of transmission; the single water-borne outbreak that has been reported[18] was in the nature of a laboratory accident.

The prevalence of human brucellosis is not known with any degree of precision. As a consequence of better diagnosis, the number of reported cases has steadily increased from 24 in 1925 to 2497 in 1937. During the period 1930–1941 29,594 cases of brucellosis were reported in the United States, an average annual rate of 1.87 per 100,000 population.[19] In 1945 a total of 44 states reported 4621 cases and 92 deaths, rates of 4.0 and 0.1

15 See, for example, Hasley: Jour. Inf. Dis., 1930, 46:430.
16 Meyer and Eddie: Jour. Inf. Dis., 1941, 68:24.
17 Evans: Pub. Health Repts., 1937, 52:295.
18 Huddleson and Munger: Amer. Jour. Pub. Health, 1940, 30:944.
19 Jordan, Borts, Harris and Jennings: Amer. Jour. Pub. Health, 1943, 33:773.

per 100,000 respectively, but the cases reported represent a minimum and Evans[2] estimates that 30,000 to 40,000 persons are ill each year with the disease. The disease is found in rural areas for the most part and the incidence in urban areas is very low. The incidence is high in the midwestern states and, in geographical distribution, tends to parallel the extent of the hog-raising industry. The incidence of brucellosis in cattle is relatively high (10 per cent?) and more or less uniform throughout the country. Swine brucellosis is not so common; about 3 per cent of hogs show significant agglutinin titers.

Bacteriological Diagnosis of Brucellosis.[20] The laboratory diagnosis of brucellosis involves the demonstration of the causative microorganism and of specific antibodies. When the bacterium can be isolated and identified the diagnosis is solidly established, but the presence of antibodies indicates only an immune response to what may have been a past rather than present infection and is, therefore, only suggestive.

Brucella is more often found in the blood stream in man, particularly in the pyrexial period, but cannot always be isolated. Blood or blood clot is added to tryptose broth in 2 to 5 ml. amounts and incubated in an atmosphere containing 25 per cent CO_2. The enrichment culture should be subcultured at four-day intervals and, if subcultures are negative, carried for a period of not less than three weeks. For subculture agar plates of liver infusion or tryptose agar should be inoculated. The bacteria may be identified by agglutination with antiserum; differentiation between *Br. melitensis* and *Br. abortus-Br. suis* may be made by agglutination with type-specific antiserum, and the culture tested for H_2S production and growth in the presence of thionine and basic fuchsin.

Agglutinins may be titrated in the usual way but the interpretation of the test may be difficult unless the titer is very high. Probably a titer below 1:80 is not significant, while titers as high or higher than 1:1000 are seldom if ever observed in the absence of infection. Even a very high titer is not conclusive evidence of infection, of course. Conversely, some infected individuals show only low agglutinin titers, perhaps 1:20. Huddleson feels that the opsonophagocytic test for the presence of opsonins is a much more reliable measure of the immune response. Citrated whole blood is mixed with bacterial suspension, incubated without agitation for thirty minutes, and a film of the mixture spread on a glass slide. The film is dried rapidly and stained with Hastings' stain (Wright's stain may be used) and examined for the presence of the bacilli in the neutrophils. The test is negative when no phagocytosis occurs, slight when 1 to 20 bacilli are found per cell (25 counted), moderate when the phagocytosed bacilli are 21 to 40 per cell, and marked when there are more than 40 per cell. In the last case, the neutrophils are usually packed with bacilli and there are too many to be counted. Marked phagocytosis in 40 per cent or less of the cells examined indicates infection, and in more than 60 per cent, an immune reaction.

[20] See the general discussion by Foshay: Amer. Jour. Clin. Path., 1940, *10*:176; and by Harris: Bull. New York Acad. Med., 1943, *19*:63.
[21] See the review by Huddleson: Bact. Rev., 1942, *6*:111.

Immunity.[21] The resistance of calves and non-pregnant cows to clinically apparent brucellosis is clearly an expression of natural immunity, though the older animals respond to the microorganism with the production of antibodies and the development of an increased resistance to subsequent infection. Man likewise appears to have a high degree of natural resistance to the infection and it is probable that there are many more infections than clinical cases of brucellosis. In the series studied by Huddleson and Munger[18] in which exposure to infection was known, only about half the individuals showing evidence of infection by an immune response had clinically apparent disease.

In man the immune response is evidenced by the appearance of agglutinins, opsonins and hypersensitivity to preparations (*brucellergen*) of the cell substance of the bacteria. It is not clear, however, that this response is associated with an increased resistance, *i.e.*, effective immunity, to the infection. Prophylactic inoculation with Brucella vaccines is not practical in man, though the available evidence indicates that it is effective in cattle. Killed vaccines have not given satisfactory results, but those of living avirulent bacilli have given encouraging results in the control of bovine brucellosis by vaccination of calves. A smooth, avirulent strain known as strain No. 19 has been the most widely used. The desirability of general vaccination is not completely agreed upon, however, since it does not eliminate infection in herds.[22] The therapeutic use of antisera or vaccines in human brucellosis has given disappointing results.

BRUCELLA BRONCHISEPTICA

This microorganism is very similar to *Br. abortus,* but is motile and highly aerobic. It does not produce hydrogen sulfide. It is immunologically related to *Br. melitensis* and *Br. abortus,* but can be separated from them by agglutinin-absorption tests. It also resembles *Hemophilus pertussis* both culturally and immunologically. Originally isolated from dogs ill with distemper, it is not now generally believed to stand in any causal relation to that disease. It is, however, frequently found as the cause of bronchopneumonia in guinea pigs and other rodents.

[22] See the discussions by Dykstra: Jour. Amer. Vet. Med. Assn., 1947, *110*:96; Crawford: *ibid.*, 1947, *110*:99; Haring: *ibid.*, 1947, *110*:103.

PASTEURELLA

Hemorrhagic Septicemia; Plague; Tularemia

The term "hemorrhagic septicemia" was applied by Hueppe to a group of highly fatal infectious diseases of the lower animals in which large and small hemorrhagic areas are found in the subcutaneous tissues, serous membranes, muscles and lymph glands, and throughout the internal organs. The causative bacteria constitute a group of closely related, biochemically inactive, non-motile, gram-negative forms showing bipolar staining. The first of these bacteria to be studied was the etiological agent of fowl cholera which Pasteur used in his early studies on immunity. Others have been described as producing hemorrhagic septicemias in various lower animals. Regarded by some as but a single species under the name *Pasteurella pluriseptica,* these bacteria are usually separated into species which differ from one another in host adaptation and minor fermentation reactions. They have been given names derived from the kind of animal in which they were found.

The species of bacteria causing the hemorrhagic septicemias of lower animals are:

Pasteurella aviseptica (Bacillus avisepticus, Bacterium avisepticum, Pasteurella avicida)—the fowl cholera bacillus. Pathogenic for birds and mammals.

Pasteurella muriseptica (Bacillus murisepticus, Bacterium murisepticum, Pasteurella muricida)—found in naturally infected wild rats and pathogenic for rabbits, guinea pigs, mice and rats but not for chickens or pigeons.

Pasteurella lepiseptica (Bacillus lepisepticus, Bacterium lepisepticum, Pasteurella cuniculicida)—the bacillus occurring in contagious nasal catarrh or "snuffles" of rabbits as a clinical or latent infection. Produces septicemia in rabbits upon parenteral inoculation. Pathogenic for chickens and mammals.

Pasteurella suiseptica (Bacillus suisepticus, Bacterium suisepticum, Pasteurella suilla)—the bacillus of swine plague. Pathogenic for mice, rabbits and birds.

Pasteurella boviseptica (Bacillus bovisepticus, Bacterium bovisepticum, Pasteurella bollingeri)—produces a hemorrhagic septicemia in cattle, hogs and horses and is found in deer and wild hogs.

So far as is known, the diseases produced by these bacteria are not ordi-

narily communicable to man. Rare cases of human infection with bacilli of this group have, however, been reported.[1] Closely related to this group and classified as species of Pasteurella are the bacilli of plague or "black death" and of tularemia or "rabbit fever." *Past. pestis* differs culturally from the hemorrhagic septicemia bacilli in that it grows in the presence of bile, does not ferment sorbitol, and does not produce indol or hydrogen sulfide. Unlike the other Pasteurella species, *Past. tularensis* requires enriched media for growth.
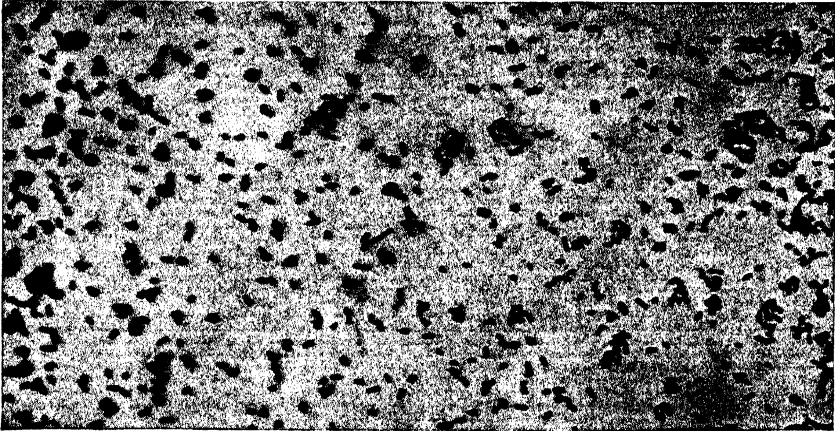
Fig. 92. *Pasteurella suiseptica.* Smear from a pure culture. Fuchsin; × 1050.

PASTEURELLA PESTIS—THE PLAGUE BACILLUS

Plague prevailed extensively throughout Europe during the Middle Ages. It has been estimated that 25,000,000 persons, or one-quarter of all inhabitants of Europe, perished in the "great mortality" or "black death" of the fourteenth century (1348–49). Few diseases have left so deep a mark on general literature. Boccaccio's *Decameron* contains one of the most vivid descriptions of the plague ever written, and Defoe's fictitious[2] *Journal of the Plague Year* provides a realistic picture of the devastation of London in 1665 by an outbreak of "black death" in which 70,000 persons perished.

For reasons that may be only partly conjectured the plague has had irregular periods of quiescence and recrudescence. Western Europe has been practically free from the plague since the middle of the eighteenth century, and the disease began its first great extension in modern times with its appearance in 1893 in Hongkong and in 1896 in Bombay. During recent years the plague has caused terrible loss of life in British India; official statistics show that in the period from 1896 to 1918 more than 10,000,000 deaths were due to this disease. In October, 1899, a case was recorded at Santos, Brazil; this is thought to be the first occurrence of the plague in the Western Hemisphere. Plague first appeared in the United States in San Francisco in 1900; it is assumed that it was introduced by infected rats from

[1] The hemorrhagic septicemia bacilli are discussed at length by Regamey: *Les infections humaines a B. bipolaris septicus* (*Pasteurelloses*). Huber, Berne. 1939.

[2] Defoe was only four years old in the year of the great plague,

the Orient. The infection apparently spread to ground squirrels and other wild rodents in the western part of the country.

The plague bacillus, *Pasteurella pestis*, was discovered almost simultaneously by Yersin and by Kitasato in 1894.

Morphology and Staining. The plague bacillus is a short, plump, ovoid rod 0.3 to 1.25 μ in length. In the body fluids the bacilli may occur in pairs, but long chains are rare and, in general, there is no characteristic arrangement. The bacilli are non-motile and are encapsulated. Involution forms are common, especially in older cultures, and coccus shapes, large rods and gigantic swollen forms may be observed. The tendency of the plague bacillus to aberrant morphology is accentuated by cultivation on media containing 3 to 4 per cent sodium chloride; the appearance of involution forms in twenty-
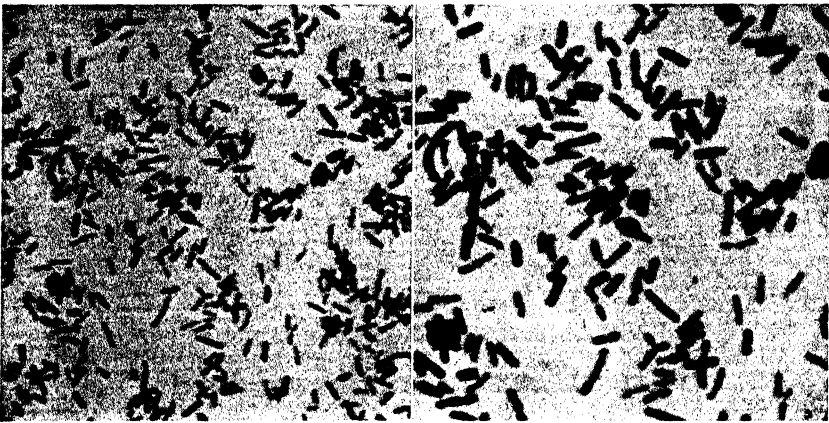
Fig. 93. The plague bacillus. Smear from pure culture; fixed in methyl alcohol and stained with methylene blue to show bipolar staining. Note the involution forms present even at twenty-four hours' incubation. Left, $\times$ 1050; right, $\times$ 1800.

four-hour cultures on salt-containing media has been regarded by some as a characteristic of differential value.

Colonies on nutrient agar or gelatin have a delicate, drop-like appearance, with a round, granular center and a thin, granular, uneven margin.

The plague bacillus is uniformly gram-negative and shows a marked tendency toward polar staining, *i.e.*, there are heavily stained areas at the ends of the cell separated by a lightly stained area in the center (see Fig. 93). For good bipolar staining the smear should be air-dried and fixed in alcohol. The usual aniline dyes, such as methylene blue, are satisfactory. The plague bacillus is best demonstrated in tissue sections by a polychrome stain.

Physiology. *Past. pestis* is not nutritionally fastidious, and growth occurs on all the ordinary culture media, although in peptone water it is very poor. Some strains will grow in amino acid media without bacterial vitamins while others require nicotinamide or thiamine or both.[3] Unlike most of the bacteria pathogenic for man, a temperature of 25° to 30° C.

[3] Berkman: Jour. Inf. Dis., 1943, 71:201; Doudoroff: Proc. Soc. Exp. Biol. Med., 1943, 53:73.

is more favorable than one of 37° C., and the limiting temperatures for growth are —2° C. and 45° C. In any case, the colonies on solid media grow slowly and never attain a large size. The plague bacillus is aerobic and facultatively anaerobic.

Sugar fermentations are variable, and a small amount of acid but no gas is produced. Neither coagulated serum nor gelatin is liquefied, and indol is not produced. Nitrates are reduced to nitrites, and a small amount of hydrogen sulfide is formed. On potato and in milk multiplication is slow and scanty; milk is rendered slightly acid but is not curdled.

One of the most characteristic cultural features is observed in the growth in broth. When the surface of this medium is covered with a layer of oil and flasks are left undisturbed for five or six days after inoculation, long, delicate filaments are formed which hang down from the surface into the depths of the clear broth, like the stalactites that depend from the roof of a grotto. Not all cultures of *Past. pestis* show stalactite growth in equal degree, and, on the other hand, a similar formation has been observed in cultures of other bacteria; the stalactite formation, therefore, while highly characteristic, especially when broth is seeded directly from fresh plague buboes, is not specific.

The plague bacillus does not exhibit any marked resistance to deleterious influences. Exposure to drying, particularly at the higher summer temperatures, kills it within a short time. The bacillus is quite sensitive to the action of sunlight and chemical disinfectants; it is killed, for example, by 0.5 per cent phenol in ten to fifteen minutes and by heating to 55° C. in about the same time. Cultures kept in the refrigerator, however, remain viable over long periods of time. In general, the life of *Past. pestis* outside the animal body is precarious, and the bacillus seems to disappear speedily from soil, water and buried cadavers.

Toxins. The toxicity of old broth cultures to experimental animals on parenteral inoculation is indicative of the toxicity of the bacillary cell substance. Baker *et al.*[4] have isolated an endotoxin by chemical fractionation of the bacilli which had an LD_{50} dose for mice of 0.6 γ and was not identical with an immunizing protein antigen. The plague bacillus also contains a factor which enhances spreading in the tissues and increases capillary premeability, and a coagulase is produced.[5]

Pathogenicity for Man. Plague in man appears most commonly in two forms, the bubonic or glandular plague and plague pneumonia. In the bubonic type the symptom-complex is characteristic, and diagnosis on clinical grounds is relatively simple. From the buboes, which may be either primary or secondary, bacilli may pass over into the blood; in fatal cases the bacteria often multiply in the blood extensively. The case fatality is 60 to 90 per cent. A primary plague septicemia can also probably occur. There are sometimes subcutaneous hemorrhages. During the plague epidemics in the Middle Ages such hemorrhages seem to have been more frequent than at present, and the dark spots to which they give rise were the origin of the popular name of "black death."

Plague pneumonia occurs secondary to the glandular infection and may

[4] Baker *et al.*: Proc. Soc. Exp. Biol. Med., 1947, 64:139.
[5] Jawetz and Meyer: Jour. Immunol., 1944, 49:15.

be transmitted to give rise to primary plague pneumonia. Pneumonic plague is usually fatal. In this variety the sputum may contain enormous numbers of plague bacilli, and the infection is spread from man to man by droplets. Because of this direct spread pneumonic plague is by far the more dangerous type. The extensive outbreak of pneumonic plague in Manchuria in 1910–12 is said to have caused approximately 60,000 deaths, the case fatality being practically 100 per cent.

A primary infection of the skin with the plague bacillus sometimes occurs (cutaneous plague) but does not seem to be common. Cases of mild plague, the so-called "pestis minor," are met with in some epidemics, but it is probable that healthy persons do not carry plague bacilli for long, if at all. The occurrence of intestinal plague in man has never been clearly established.

Epidemiology. Plague is primarily a disease of rats and other rodents and is spread from rat to rat and from rat to man through the bite of infected
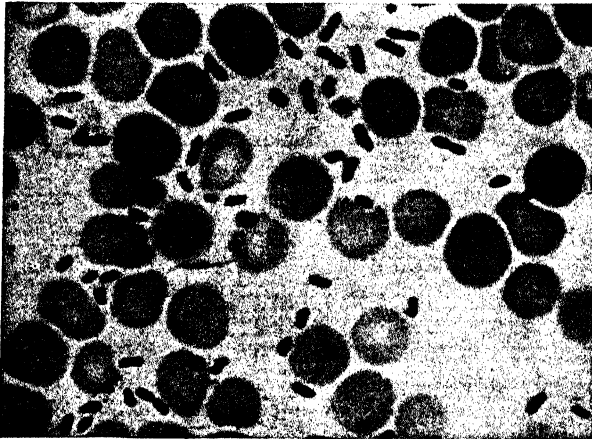
Fig. 94. The plague bacillus in the blood of an infected mouse. × 2000 (Douglas and Wheeler).

fleas. At least three species of rats, *Epimys norvegicus,* the common gray sewer rat; *E. rattus,* the black house rat and ship rat; and *E. alexandrinus,* the Egyptian rat, are known to be capable of receiving infection. The Indian rat flea, *Xenopsylla cheopis,* is probably the most important rat flea in the dissemination of plague, but *Ceratophyllus fasciatus,* the common rat flea of North America and Europe, may also transmit the disease.

The black rat lives in close association with man, and its presence in dwelling houses makes it a particularly dangerous vector of plague. It is commonly believed that the great prevalence of the plague in Europe in the Middle Ages was caused by the advent of the black rat brought in from Asia Minor in the ships of the Crusaders. The black rat in turn was largely driven out of Europe by the arrival of the fiercer gray rat from the north. This second change in the character of the rat population is thought to explain in large part the practical disappearance of the plague from Europe. Probably the advances made over the medieval type of dwellings with their "rush-strewn floors" were also a factor.

Although both the feces and the urine of infected rats sometimes contain plague bacilli, the infection is not transmitted from animal to animal under experimental conditions in the absence of fleas. The blood, however, often contains enormous numbers of the bacilli—as many as 100 million per ml. have been found—and the flea becomes infected through feeding on this infectious blood. When the plague bacilli are taken into the stomach of the flea they undergo multiplication but do not penetrate into the other parts of the body. On microscopic examination large masses of bacilli may be seen in the infected flea; the development of the infection is illustrated
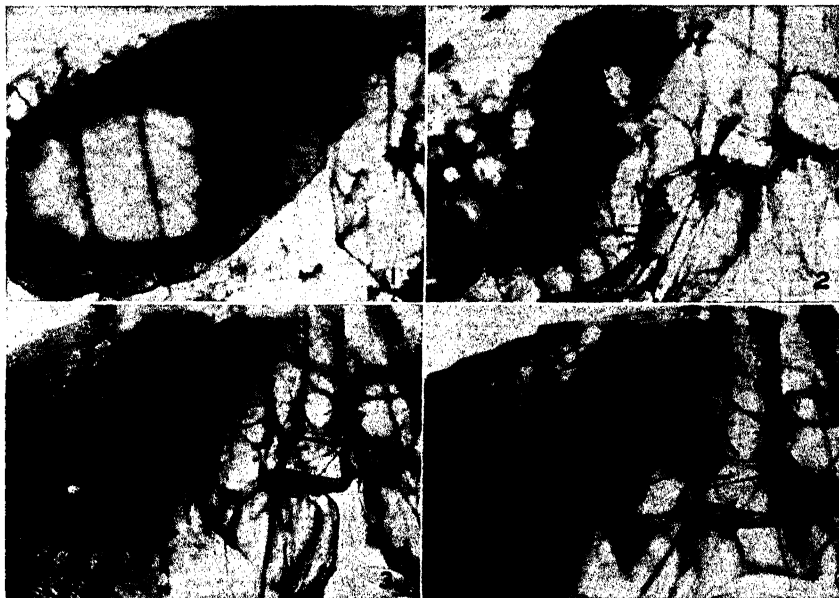
Fig. 95. Plague bacilli in the infected flea; sections stained with methylene blue. The bacilli appear as dark stained masses. *1*, Flea on the ninth day after infection. *2*, Eighteenth day after infection; note the stomach and proventriculus packed with bacilli. *3*, Twenty-second day after infection; note the large mass of bacilli and the swollen proventriculus. *4*, Twenty-third day after infection; the proventriculus is further enlarged. (Douglas and Wheeler.)

in Fig. 95 from Douglas and Wheeler.[6] The bacilli are discharged with the feces, and human infections may result from rubbing these fecal droplets into the bite as it is scratched. In some infected fleas the bacilli multiply so rapidly that they mechanically obstruct the proventriculus to such an extent that little or no food may pass. In the effort to feed, some plague bacilli are mixed with the drawn blood of the host and regurgitated into the bite, thus bringing about infection. Although rats may be infected by feeding on the carcasses of their plague-infected comrades, it is probable that, under natural conditions, transmission from rat to rat is almost always through the agency of infected fleas. It may be noted that plague and tularemia are the only bacterial diseases that are transmitted by in-

[6] Douglas and Wheeler: Jour. Inf. Dis., 1943, 72:18.

fected insect vectors (as differentiated from mechanical transmission by insects); the gut of most blood-sucking insects is strongly bactericidal and bacteria do not persist there more than a few hours. *Past. pestis* and *Past. tularensis*, however, are resistant to this bactericidal activity and the insect vectors may remain infective for days and perhaps weeks.

Plague is also present in other rodents. In California the native ground squirrels have proved highly susceptible to infection, and through the agency of these animals a great reservoir of infection termed *sylvatic plague* has developed.[7] The infection may be overt and occur in epizootic form, and it may also persist as an inapparent latent infection.[8] The squirrel
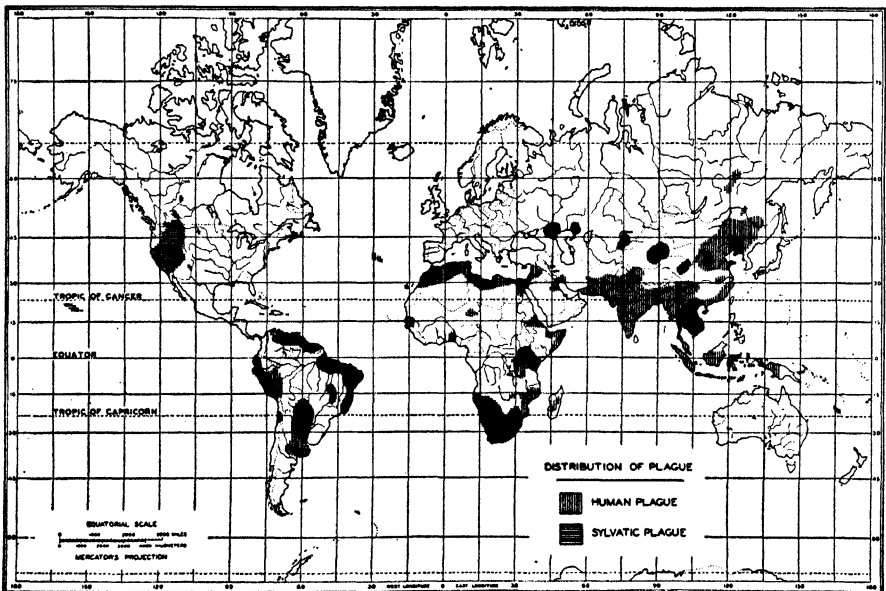
Fig. 96. The world-wide distribution of human and sylvatic plague. Redrawn from map prepared by Army Medical Intelligence, 1943. (Based on Goode *Base Map*. No. 201M. By permission of the University of Chicago Press.)

flea, *Ceratophyllus acutus*, transmits the infection to man. In other parts of the world other rodents are affected and are the means by which the disease is maintained and spread. In South Africa it is the gerbille, in Transbaikalia the tarbagan, and in Russia the spermophile that keeps the plague alive in animal reservoirs of infection; there is a focus of sylvatic plague on the Peruvian-Ecuadorian frontier in wild rodents. The tendency of the plague bacillus to establish these reservoirs in wild rodents constitutes a danger not fully appreciated. There is reason to believe that rodent infection is extending in several parts of the world, and there is also some experimental evidence that plague infection of man contracted from wild rodents is especially likely to assume the deadly pneumonic form.

[7] Plague in the western part of the United States is considered in detail by Eskey and Haas: Pub. Health Bull. No. 254, 1940.
[8] Meyer *et al.*: Jour. Inf. Dis., 1943, 73:144.

As pointed out above, plague may assume two epidemiological forms. As pneumonic plague it is transmissible by droplet infection and its epidemiological behavior is very similar to that of the other respiratory infections. As bubonic plague it is derived from an infected rodent through the agency of the flea. In its epidemic form, bubonic plague is nearly always associated with the disease in the black rat. Infections derived from other rodents, such as the California ground squirrels, are sporadic rather than epidemic. The dissemination of bubonic plague in epidemic form, then, is determined by the closeness of the association between the infected rat population and the human population. Filth and poverty associated with a large rat population provide opportunities for transmission of the disease, and if transmission takes place on a large scale an epidemic may flare up and perhaps extend to even greater proportions as the pneumonic form develops.

Bacteriological Diagnosis of Plague. In man the bacilli are found in material aspirated from buboes, in cultures or smears of internal organs, especially spleen, and, in pneumonic plague, in the sputum. The presence of gram-negative, bipolar staining, ovoid bacilli is highly suggestive. Blood cultures, taken late in the disease, should be cultured first in broth. Other material may be inoculated directly on blood agar and glycerol agar. Cultures may be identified by cultural and biochemical characteristics and by agglutination in plague antiserum. The bacilli show some tendency to spontaneous agglutination and the slide agglutination test is unsatisfactory. Guinea pigs may be inoculated subcutaneously or, with specimens that have undergone gross contamination and decomposition, by rubbing the material on the freshly shaven abdomen; the plague bacilli penetrate the minute abrasions while the contaminants do not. The animals die in two to five days; postmortem findings are characteristic and include subcutaneous and general congestion, congested spleen, granular liver and pleural effusion. The bacilli may be found in spleen smears and elsewhere and cultured. It is important that the animal be freed of ectoparasites before inoculation. Plague in rodents may be diagnosed by postmortem findings, which are similar to those in the guinea pig, by microscopic and cultural demonstration of the bacilli, and by guinea pig inoculation.

Immunity. Recovery from plague confers a solid immunity to subsequent infection. Experimental animals may be immunized by inoculation with suspensions of attenuated or killed plague bacilli, and numerous attempts have been made to actively immunize man in this way. One of the first plague vaccines was that of Haffkine and consisted of heat-killed bacilli from old cultures. This and other killed vaccines have never been particularly satisfactory and do not produce an efficacious immunity in man. The vaccine used by the United States Army during World War II consisted of a suspension of 2000 million formalin killed virulent plague bacilli per ml., and was given in two doses, 0.5 ml. and 1.0 ml., seven to ten days apart. Inoculation with living attenuated bacilli produces a much more solid immunity in experimental animals, however, and avirulent strains appear to be as efficient immunizing antigens as virulent strains.[9] The use of living attenuated cultures for human inoculation has been attempted in the past

[9] Pirie and Grasset: South African Med. Jour., 1938, *12*:294; *ibid.*, 1941, *15*:275.

but not continued because of possible attendant danger. The use of such vaccines in man has been investigated by Grasset[10] in South Africa with encouraging results, and a similar vaccine has been used in recent years in the Netherlands Indies in more than ten million inoculations without untoward results.[11]

Antisera to the plague bacillus may be prepared by the judicious immunization of horses, or other animals, but their efficacy as therapeutic agents is doubtful.

PASTEURELLA PSEUDOTUBERCULOSIS

Past. pseudotuberculosis (*Bacillus pseudotuberculosis rodentium*) causes a disease of rodents, particularly guinea pigs. It resembles *Past. pestis* very closely but, unlike the latter, is usually actively motile at 22° C. Other differential marks are its tendency to produce alkali in milk cultures and its relatively low pathogenicity for white rats. The natural mode of infection is probably by way of the alimentary tract. Subcutaneous inoculation of guinea pigs proves fatal in two to three weeks, with caseous swellings and nodules ("pseudotubercles," which have unfortunately given this bacterium its name) in various organs. *Past. pseudotuberculosis* has been found, though rarely, in animals other than the guinea pig, and a few cases of infection have been reported in man.

PASTEURELLA TULARENSIS

Tularemia is a disease of rodents, rabbits in particular, that is transmitted to man either directly through the handling of the flesh of infected animals or indirectly through an insect vector. *Past. tularensis* (*Bacterium tularense*) was discovered by McCoy and Chapin[12] in a plague-like disease of the California ground squirrel. Tularemia in man, however, is contracted largely from the rabbit and it was shown by Francis[13] that the disease known in Utah as deer fly fever is, in fact, tularemia transmitted from infected rabbits to man by the bite of the fly *Chrysops discalis*. Francis also found that *Past. tularensis* was present in rabbits sold in the markets of Washington, D. C., and that a disease known as rabbit fever was not infrequent among those in contact with the rabbits. In 1938 2088 cases with 139 deaths and in 1939 2200 cases with 150 deaths were reported. Human cases have been observed in all forty-eight states and in the District of Columbia.[14]

Morphology and Staining. In culture *Past. tularensis* is a minute, gram-negative pleomorphic rod 0.2 μ in breadth and 0.3 to 0.7 μ in length; the coccoid form predominates in young cultures and the bacillary form in older cultures. In smears from the spleens of infected mice or guinea pigs the bacteria appear as coccoid forms in well-defined clusters. Capsules are present in the body; spores are not formed, and the microorganisms are

---

[10] Grasset: Trans. Roy. Soc. Trop. Med. Hyg., 1946, 40:275.
[11] *Cf.* Otten: Mededeel Dienst Volksgezondheid Nederland-Indië, 1941, 30:61.
[12] McCoy and Chapin: Jour. Inf. Dis., 1912, 10:61.
[13] Francis: Pub. Health Repts., 1919, 34:2061; *ibid.*, 1923, 38:1391, 1396.
[14] *Cf.* Pub. Health Repts., 1940, 55:667.

non-motile. According to Hesselbrock and Foshay[15] it reproduces by a number of methods, including binary fission, budding, filament formation and the like, and they regard it as closely related to the microorganisms of the pleuropneumonia group (p. 547).

On solid media *Past. tularensis* forms minute, transparent, droplike colonies that are mucoid in consistency and readily emulsifiable.

This bacterium is somewhat difficult to stain; methylene blue is not satisfactory, but either carbol fuchsin or aniline gentian violet may be used. Bipolar staining may be observed.

Physiology. *Past. tularensis* differs sharply from the other members of this genus in that it will not grow on the ordinary media. It may be cultured on a coagulated egg-yolk medium or on blood dextrose cystine agar. Until recently it has been assumed that this bacterium could not be grown on a liquid medium. Tamura and Gibby[16] have found, however, that
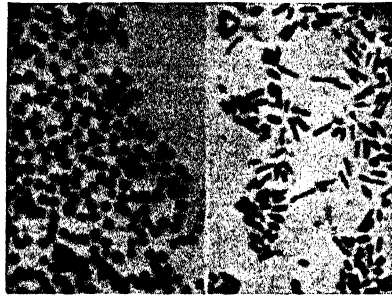
Fig. 97. *Pasteurella tularensis.* Note change from coccoidal to bacillary form in twenty-four hours on fresh culture medium (Francis).

growth occurs in casein or gelatin hydrolysate medium supplemented with biotin, blood cell extract and liver extract. Steinhaus and McKee[17] have found that a medium of heart infusion, dextrose, cystine and hemoglobin will support good growth. It is aerobic and facultatively anaerobic, and its optimum temperature is 37° C. Fermentation reactions have been investigated in some detail by Francis[18] who has found that glucose, maltose and mannose are fermented, the fermentation of glycerol, levulose and dextrin is irregular, and mannitol, galactose, xylose, trehalose, salicin, arabinose, adonite, sucrose, lactose, amygdalin, dulcitol, erythritol, inositol, inulin, raffinose, sorbitol and rhamnose are not fermented. Differential fermentations are of no value. It is killed by exposure to 56° C. for ten minutes. It has been reported that this bacillus contains an endotoxin.

Pathogenicity. Two clinical types of tularemia are recognized, one the glandular or ulceroglandular type, which is the more common, the other the so-called "typhoidal" type. In the first instance the acute stage of the disease is characterized by headache, pains and fever, and a papule appears, fre-

[15] Hesselbrock and Foshay: Jour. Bact., 1945, 49:209.
[16] Tamura and Gibby: Jour. Bact., 1943, 45:361.
[17] Steinhaus and McKee: Pub. Health Repts., 1944, 59:78.
[18] Francis: Jour. Bact., 1942, 43:343.

quently on a finger where presumably the bacilli enter the body, which
later breaks down and forms an ulcer. The axillary and epitrochlear glands
become painful and swollen and may break down with the discharge of
purulent material. In cases infected via the conjunctiva, ulcers form on
the inner surfaces of the eyelids and the cervical and pre-auricular glands
may become tender and somewhat swollen. In the typhoidal type of the
disease there are no local symptoms.

During the first week of illness the bacilli may be present in the blood
and have been cultured, although in general, cultures made directly from
man are not successful and the bacillus is best isolated by guinea pig inocu-
lation and culture from necrotic foci found in liver, spleen and lungs of the
pig on autopsy. Direct cultivation from the blood is rarely possible but has
been accomplished, and it has been suggested that during the first week of
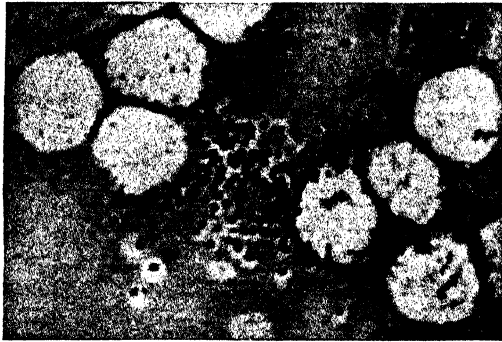
Fig. 98. *Pasteurella tularensis;* coccoidal form; blood of rabbit (Francis).

the disease an initial bacteremia may occur which, in fulminating cases,
develops into septicemia. The bacilli can only rarely be found in smear
preparations from human cases. In the experimental disease the bacilli are
present in the lymph spaces and phagocytic cells of infected tissues, and
their presence within the cells in enormous numbers has caused some work-
ers to suggest an intracellular proliferation of the microorganisms similar
to that of the rickettsiae (Chapter 35). Agglutinins are present in the blood
in the second week of the disease and may persist in diminishing amounts
for at least as long as eighteen years after recovery. The average duration
of the disease is two to four weeks. The case fatality is low, 4.8 per cent,
and the pathology of the disease in man is not well known.[19]

A variety of lower animals have been found to be naturally infected—in
addition to the ground squirrels and rabbits noted above, wild rats and
mice, woodchucks, opossums, beavers, coyotes, deer, red foxes, ground hogs,
muskrats, hogs, skunks, dogs, cats and lambs. The infection also occurs
naturally in some birds such as sage hens, grouse and quail. The guinea
pig is highly susceptible to artificial inoculation.

Epidemiology. As indicated above, tularemia is acquired by man from

[19] For postmortem findings *cf.* Mathews: New Orleans Med. and Surg. Jour., 1938,
90:479.

lower animals either directly or indirectly. The bacilli may enter the un-broken skin of the guinea pig, and possibly this may occur in man through the dressing of infected rabbits and other animals, or the microorganisms may enter by means of minute abrasions on the skin of the hand. Eye infection occurs not infrequently; in fact, such infections were the first human infections with the bacillus observed. Over 90 per cent of the human cases in this country are contracted from rabbits and it is estimated that about 1 per cent of wild rabbits are infected. Jellison and Parker[20] have reported that the cottontail rabbit, species of Sylvilagus and *S. flori-danus* in particular, is by far the most important source of infection in this country, accounting for more than 70 per cent of all human cases in North America. Many wild animals are naturally infected and Burroughs *et al.*[21]

Fig. 99. *Pasteurella tularensis* in hepatic cells of mouse (Francis).

have compiled a list of forty-eight naturally infected vertebrates. Laboratory infection is not uncommon; 56 cases acquired from dissection were reported to 1940. *Past. tularensis* has also been found in streams and is perhaps asso-ciated with the epizootics occasionally observed in beavers. Present evidence indicates that fish cannot be infected with *Past. tularensis* and probably play no part in the infection of water.[22] Water-borne epidemics have occurred in Russia and Turkey but none has been reported in this country.

The transmission of tularemia by an insect vector is common. In addi-tion to the deer fly, *Chrysops discalis, Dermacentor andersoni, D. variabilis, D. occidentalis, Hemaphysalis leporis palustris, H. cinnabarina* and *Ixodes ricinus californicus* may carry it. In all probability the wood ticks serve to disseminate the infection in the animal population, and it is of particular interest that the infection is transmitted from the adult tick to the egg and both the larvae and the nymphs are infectious. Tularemia may, then, be in part maintained in the insect population.

20 Jellison and Parker: Amer. Jour. Trop. Med., 1945, 25:349.
21 Burroughs *et al.*: Jour. Inf. Dis., 1945, 76:115.
22 Morgan: Amer. Jour. Trop. Med., 1947, 27:399.

Tularemia has been found in all parts of the United States, in Japan and in Central Europe,[23] and extensive epidemics have occurred in Russia.

Bacteriological Diagnosis of Tularemia. As indicated above, *Past. tularensis* is difficult to cultivate from infected material. Specimens should be streaked on blood-dextrose-cystine agar. Characteristic minute, drop-like colonies may appear in three to five days but a culture should not be recorded as negative in less than three weeks. The procedure of choice is the intraperitoneal inoculation of a guinea pig with a saline emulsion of the specimen; relatively large amounts are required, usually 4 to 8 ml., of a fairly heavy emulsion. The animal should die in five to ten days. The pathology is characteristic and includes a hemorrhagic edema without pus at the site of inoculation, enlargement of the cervical, axillary and inguinal lymphatics which contain dry caseous material, and small white necrotic areas in the liver and spleen. Smears and cultures may be made but not infrequently the bacilli cannot be found or cultivated. In such instances the diagnosis depends on the guinea pig pathology.

Isolated cultures may be identified by specific agglutination. Conversely, patient's serum may be tested for agglutinins; a titer of 1:80 or higher is usually regarded as diagnostic if there are no Brucella agglutinins. In the event that Brucella is also agglutinated, the agglutination of *Past. tularensis* occurs more rapidly and to higher titer in tularemia.

Immunity. An attack of tularemia confers a solid immunity, and second infections, when they occur, produce only a local lesion. It may be noted that antibodies (agglutinins) to *Past. tularensis* show some cross-reaction with *Brucella melitensis* and *Brucella abortus,* and for this reason, together with the complex nutritive requirements of these bacilli, some workers have placed them with the Brucella group and termed them *Br. tularensis.*

Prophylactic inoculation with vaccines is not successful in experimental animals in that it does not protect against the injection of virulent strains. There is some evidence, however, that vaccine prophylaxis in man confers a useful degree of protection.[24] The value of therapeutic antisera is doubtful; its use has been advocated by some[25] but others[26] regard it as having no significant effect.

[23] Perm. Com. Office Int. Hyg. Pub., Session, May, 1937. *Cf.* Bull. Hyg., 1937, *12:*675.
[24] Foshay, Hesselbrock, Wittenberg and Rodenberg: Amer. Jour. Pub. Health, 1942, 32:1131.
[25] Foshay: Medicine, 1940, *19:*1.
[26] Francis and Felton: Pub. Health Repts., 1942, *57:*44.

Chapter 25

THE HEMOPHILIC BACTERIA

The genus Hemophilus as at present constituted is a heterogeneous one. The true hemophilic or hemoglobinophilic bacteria are those whose growth necessitates or is especially favored by the presence of hemoglobin in the culture medium. The inclusion of bacteria other than these nutritionally distinctive types in the genus Hemophilus is questioned by many. Bacteria such as the bacillus of whooping cough and the Morax-Axenfeld and Ducrey bacilli are, then, to be associated with the truly hemophilic forms such as Pfeiffer's bacillus in only a tentative way pending a more satisfactory grouping.

THE HEMOPHILIC GROUP

Species	Growth Requirements		Hemolysis
	X Factor	V Factor	
H. influenzae	+	+	−
H. hemolyticus	+	+	+
H. parainfluenzae	−	+	±
H. suis (influenzae suis)	+	+	−
H. canis (hemoglobinophilus)	+	−	−
H. pertussis	−	−	+
H. duplex (Morax-Axenfeld)	−	−	±
H. ducreyi	−	−	+

HEMOPHILUS INFLUENZAE (PFEIFFER'S BACILLUS)

Hemophilus influenzae was isolated by Pfeiffer in 1892, and was until relatively recently regarded by many as the etiologic agent of epidemic influenza. Influenza has, however, been shown to be caused by a filterable virus (p. 865), and the name influenzae has no etiological significance.

Morphology and Staining. Pfeiffer's bacillus is one of the smallest known pathogenic bacteria, rarely exceeding 1.5 μ in length and 0.3 μ in

thickness. The ends of the cell are rounded, capsules are not generally observed but are present in smooth cultures, spores are not formed, and the bacillus is non-motile. There is a marked tendency to produce threads and other anomalous forms in culture which is, to some degree, a characteristic of strains. Some workers have attempted to differentiate varieties on the basis of morphology, but there is a continuous series of types, ranging from predominantly coccobacillary forms to predominantly longer bacilli and threads, and no sharp distinction can be made. There is a tendency, however, to regard the coccobacillary forms as "typical" and the longer forms as "atypical"; the typical form appears to predominate in strains isolated from pathological processes.
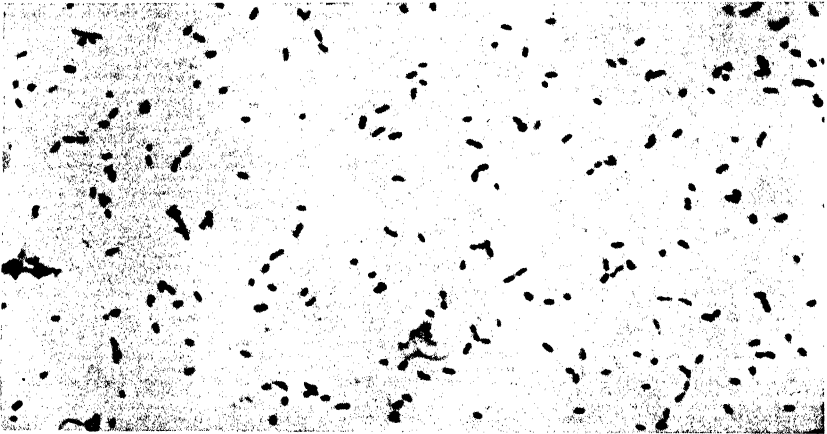
Fig. 100. *Hemophilus influenzae*, pure culture. Note the variability from coccoid to bacillary form and the presence of longer filaments. Fuchsin; × 1050.

On blood agar the colonies of Pfeiffer's bacillus are very small, rounded, discrete and transparent and may reach the size of a small pinhead. If the culture is contaminated with other microorganisms, especially *Staphylococcus aureus,* the colonies are considerably larger, more opaque, and of a grayish white color, and develop most luxuriantly in the neighborhood of the foreign colony, a phenomenon termed the "satellite phenomenon."

These bacilli are somewhat more difficult to stain than most bacteria; Löffler's methylene blue for five minutes or dilute (1:10) carbol fuchsin for ten minutes is satisfactory. They are gram-negative.

Physiology. One of the more fastidious bacteria, Pfeiffer's bacillus requires, as noted above, the presence of blood in the culture medium. It has been found that two substances present in such blood media are necessary to the growth of this bacterium; one, designated as the "X factor," is heat-stable and associated with hemoglobin, and the other, the "V factor," is heat-labile and is found in yeast and various vegetable extracts as well as in whole blood. The satellite phenomenon noted above is due to the formation of the V factor by other bacteria and its diffusion into the medium from the colony. The X factor is replaceable by hemoglobin or hematin.

Granick and Gilder[1] have shown that the iron protoporphyrin can be replaced with certain other iron porphyrins, but not by porphyrins such as meso-, hemato-, deutero- and coproporphyrins which, however, suppress growth by a competitive inhibition, and such activity is eliminated by methylation of the propionic acid side chains. It seems that these or related compounds are required for respiration—some strains do not require it for anaerobic growth. It has been suggested that hematin is required for the synthesis of catalase; hematin can be replaced by cysteine which would reduce peroxide and make catalase unnecessary. The V factor may be replaced by coenzyme I or coenzyme II but not by nicotinic acid or its amide. Apparently the whole coenzyme molecule must be supplied and it is assumed that the thermolabile substance is of this nature (see p. 118).

A number of media have been devised for the cultivation of the influenza bacillus. Avery's oleate hemoglobin agar, prepared by the addition of sodium oleate and a suspension or erythrocytes to an infusion agar base, is one of the best of these, for not only is the growth of *H. influenzae* enhanced but the growth of streptococci and pneumococci and some other bacteria present in the sputum and nasal mucus is inhibited. The influenza bacillus grows luxuriantly on chocolate agar, prepared by the addition of fresh blood to hot (90° C.) infusion agar, and heavy growths for agglutination and other purposes may be obtained, but this medium does not differentiate and hence is not especially suitable for primary isolation. Fildes' agar, an infusion agar base to which has been added a peptic digest of blood, supports good growth of the influenza bacillus and is used especially by British workers.

No growth occurs on gelatin or potato. Milk, containing blood, is rendered slightly alkaline by some strains. Nitrate is reduced to nitrite. Some strains—about 50 per cent—form indol. Fermentation reactions are variable, some strains being inactive while others ferment dextrose and other carbohydrates. Some strains are hemolytic while others are not. The hemolytic strains of *H. influenzae* do not appear to be clearly marked from the non-hemolytic strains by any other differential characters, since indol production and carbohydrate fermentation occur in both groups.

The influenza bacillus shows little resistance toward external conditions. Desiccation is quickly fatal. A pure culture suspended in water and then dried on silk threads loses its vitality within twenty-four hours; in dried sputum life is maintained somewhat longer, but not, as a rule, beyond forty-eight hours. The bacilli are readily killed by disinfectants. Even under favorable conditions artificial cultures soon die out, and in order to preserve vitality subcultures must be made every four or five days; chocolate agar is suitable for maintaining stock cultures.

Varieties. Subdivisions of *H. influenzae* have been made by a number of workers, but whether such varieties deserve the dignity of species standing accorded some of them by the Bergey (1948) classification is open to serious question. The morphologically "typical" and "atypical" forms have been noted above, but these are not regarded as separate species. Other distinctions which have been made on the basis of hemolysis and nutritive requirements may be summarized briefly:

[1] Granick and Gilder: Jour. Gen. Physiol., 1946, 30:1.

(1) The hemolytic and non-hemolytic varieties. The non-hemolytic form is designated *H. influenzae* by Bergey and the hemolytic form *H. hemolyticus*. Both require the X and V nutritive factors. These are generally regarded as a single species, *H. influenzae*.

(2) The swine influenza bacillus, *H. influenzae suis* or *H. suis*, which closely resembles Pfeiffer's bacillus except that it is relatively inert biochemically and differs immunologically. This bacterium, in association with a filterable virus, is causally associated with swine influenza. Both X and V factors are required for growth.

(3) The para-influenza bacilli, *H. parainfluenzae*, which closely resemble *H. influenzae* except that only the V factor is required for growth and these bacteria may be cultivated on agar containing serum or ascitic fluid. Although these bacilli are defined as non-hemolytic, hemolytic strains showing the same nutritive requirements are found.

(4) *H. canis* (*H. hemoglobinophilus, H. hemoglobinophilus canis*), found in the preputial secretions of dogs. It closely resembles *H. influenzae* except that it requires only the X factor for growth.

Variation and Antigenic Structure. As tested by direct agglutination, *H. influenzae* is antigenically heterogeneous. Pittman,[2] however, has described rough and smooth forms in which the smooth form is encapsulated. By means of the agglutination test the encapsulated bacilli are found to fall into five immunological types, designated A, B, C, D, E and F, and the antigens responsible are specific polysaccharides.[3] Diagnostic and therapeutic antisera may be prepared for each of these types.[4] It appears that many strains isolated are in the rough form, and these, by the agglutination test, are immunologically heterogeneous. Antigenic proteins may be extracted from the cell substance of influenza bacilli.[5] One of these, fraction M, was found to be common to most strains of *H. influenzae*, indicating an immunological homogeneity demonstrable by precipitin tests with appropriately prepared antigens. It will be clear that the antigenic structure of *H. influenzae* is by no means fully understood as yet. The influenza bacilli appears to be immunologically related to certain of the pneumococcus types; Type A shows cross-reactions with pneumococcus Type 6b and Type B cross reacts with pneumococcus Type 6 and Type 29. The immunological relationship of other Hemophilus species to the influenza bacillus and to one another is not yet known.

The S-R dissociation noted by Pittman is to some degree reversible by growth in the presence of anti-R immune serum. The relation of virulence to this dissociative change is not known.

Toxins. As in the case of many other bacteria, the cell substance of the influenza bacillus is toxic to experimental animals, mice in particular, upon parenteral inoculation. Toxic substances are produced in fluid cultures, are filterable, and may appear in appreciable quantities after six to eight hours'

---

[2] Pittman: Jour. Exp. Med., 1931, 53:471.
[3] For the preparation of these see MacPherson, Heidelberger and Alexander: Jour. Immunol., 1946, 52:207.
[4] See Alexander, Leidy and MacPherson: Jour. Immunol., 1946, 54:207.
[5] Platt: Australian Jour. Exp. Biol. Med., 1939, 17:19.

incubation. Relatively large quantities of the filtrate (2 to 4 ml.), however, are necessary to produce death in rabbits, and it is probable that a true exotoxin is not formed.

Pathogenicity for Man. The pathogenicity of Pfeiffer's bacillus for man is shown by the occurrence of cases of meningitis, mostly in infants and of a high fatality, in which *H. influenzae* is found in pure culture in the cerebrospinal fluid. These cases of "influenzal meningitis," while not very numerous, show that certain strains of this microorganism possess a definite invasive power. *H. influenzae* meningitis is the fourth commonest form of purulent meningitis; it occurs most often in the second six months of life and the case fatality rate is between 90 and 100 per cent. Occasional cases of otitis media, appendicitis, sinusitis and other localized infections may be caused by this bacterium.

In infections of the respiratory tract the influenza bacillus is frequently present, and it is found on autopsy in pneumonic lesions under conditions where its destructive action upon the tissues can hardly be doubted. Whether it is present as a primary or secondary invader in these cases is more uncertain. Its common occurrence in diseases like measles, whooping cough and tuberculosis indicates that its growth on human tissue is favored by the presence of other infecting agents. It seems probable that in respiratory infections *H. influenzae* commonly follows in the wake of some other microorganism.

It may be noted that the relation of this bacillus to influenza is very likely that of a secondary invader to the initial virus infection. It is frequently but not invariably present in cases of influenza and, of course, occurs in the absence of this disease. In the case of swine influenza, however, the influenza bacillus is, in association with a filterable virus, causally related to the disease (p. 869).

Pathogenicity for Lower Animals. Except in swine influenza, the influenza bacillus is probably not a natural pathogen of lower animals. Upon intraperitoneal inoculation into laboratory animals, mice, guinea pigs and rabbits, large doses of these bacteria produce death within one or two days. Whether this is an actual invasion rather than a toxemia is uncertain; the bacilli may be found in the peritoneal exudate but usually not in the heart's blood, and petechial hemorrhages may be observed scattered over the peritoneum and, sometimes, the pleura. Certain strains produce a fatal infection in mice on intracerebral inoculation. As tested by intraperitoneal inoculation, the virulence of *H. influenzae* varies greatly from strain to strain; the virulent strains are in a minority and strains from influenzal meningitis are generally among the more virulent.

By inoculation of the mucous membrane of the upper respiratory tract of normal monkeys Blake and Cecil[6] succeeded in producing an acute upper respiratory disease resembling influenza with pathologic changes similar to spontaneous, uncomplicated *H. influenzae* pneumonia in man. Inoculation experiments upon man with pure cultures of these bacilli, however, have given a surprisingly large number of negative results.[7]

Other hemophilic bacteria have been described in connection with diseases

[6] Blake and Cecil: Jour. Exp. Med., 1920, 32:691, 719.
[7] *Cf.* Rosenau: Jour. Amer. Med. Assn., 1919, 73:311; McCoy and Richey: Pub. Health Repts., 1919, 34:33.

of lower animals. *H. canis,* for example, has been found in association with inflammation of the prepuce in dogs but is apparently harmless. Other hemophilic bacilli such as *H. bovis, H. gallinarum, H. muris, H. ovis* have been isolated from lower animals. Their relationship to *H. influenzae* and other better known species is not clear.

THE KOCH-WEEKS BACILLUS

A small bacillus, first observed by Koch in 1883 in a series of eye inflammations in Egypt, was successfully cultivated by Weeks in New York in 1887, and is now recognized as the cause of a world-wide and highly contagious form of conjunctivitis sometimes known as pink-eye.

It has been stated that the Koch-Weeks bacillus will grow on serum agar, or a mixture of glycerol agar and ascitic fluid or, at times, even on nutrient
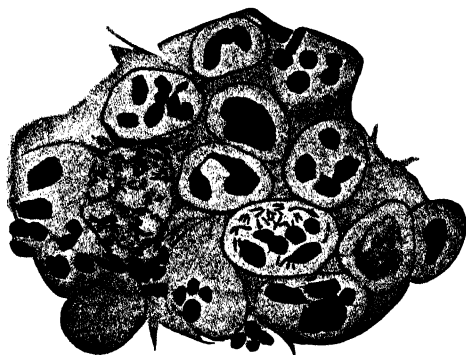
Fig. 101. Koch-Weeks bacillus in conjunctivitis; × 900 (Axenfeld, Kolle and Wassermann).

agar. Others have found, however, that both the X and V factors required by *H. influenzae* are required by the Koch-Weeks bacillus. It is, in fact, highly probable that this bacillus is identical with *H. influenzae.*

The bacilli possess slight powers of resistance, and it is unlikely that dust is a means of conveying the infection. Direct contact with infective material through the medium of hands, towels, handkerchiefs, etc., is the usual mode of transmission. A peculiar form of hand infection due to an organism probably identical with the Koch-Weeks bacillus has been described.

HEMOPHILUS PERTUSSIS

Bacilli resembling *H. influenzae* were reported by early observers as occurring in a large proportion of cases of whooping cough. Although there are minor differences in the descriptions of these organisms as given by different observers, the cultural and morphological characters are essentially similar, and there seems little doubt that Spengler, Jochmann and Kraus, Wollstein, and Davis discovered the same bacillus. More definite results were obtained by Bordet and Gengou[8] who found in the bronchial exudate from cases of whooping cough a characteristic short oval bacillus which grew

[8] Bordet and Gengou: Ann. Inst. Pasteur, 1906, 20:731.

feebly on a special medium they devised. Earlier named *Bacillus pertussis,* this microorganism is now known either by that name or as *Hemophilus pertussis,* or, more casually, as the Bordet-Gengou bacillus.

Morphology and Staining. The Bordet-Gengou bacillus is a small ovoid rod from 1.0 to 1.5 μ in length and 0.3 to 0.5 μ in breadth. The majority of the bacteria occur singly, although they may occasionally be seen in pairs end to end; chains do not occur in smears of bronchial exudate, but short chains may be seen in cultures in liquid media. This morphology is relatively constant and there is not the tendency to the formation of thread-like and other aberrant forms which is exhibited by the influenza bacillus. *H. pertussis* is non-motile and non-spore-forming, and the smooth form is encapsulated.
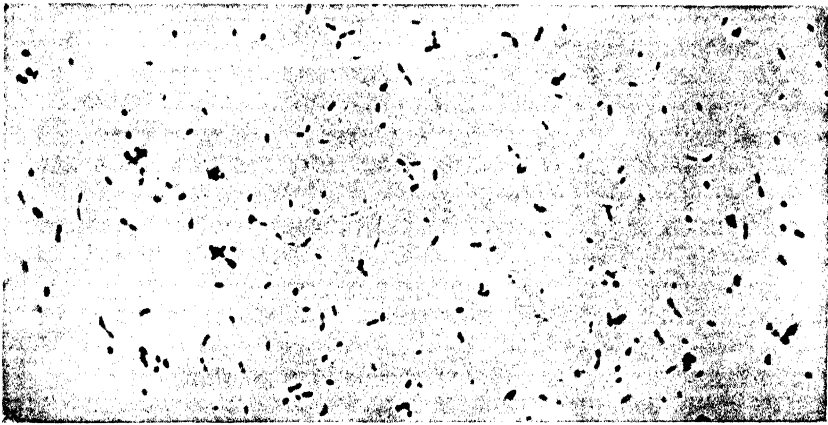
Fig. 102. *Hemophilus pertussis,* pure culture. Fuchsin; $\times$ 1050.

On the Bordet-Gengou medium the colonies are smooth, raised and glistening, with a metallic or pearl-like luster, and are larger and more opaque than those of the influenza bacillus. Forty-eight to seventy-two hours' incubation is required for their appearance and development. Upon further incubation they acquire a slight brownish color. A mucoid substance is abundantly produced by the culture, and the growth is sticky and tenacious. On blood agar the colonies are surrounded by a narrow zone of hazy hemolysis.

The bacilli stain with a little difficulty and, as in the case of the influenza bacillus, methylene blue or dilute carbol fuchsin must be applied for five to ten minutes; carbol toluidine blue is recommended by some workers, and stains the bacilli a lilac color. A tendency to bipolar staining may be observed. They are gram-negative.

Physiology. *H. pertussis* is difficult to cultivate upon primary isolation, and the Bordet-Gengou medium upon which it grows readily consists of 1 per cent glycerol agar or glycerol broth made with macerated potato and added to an equal volume of human or rabbit blood. By repeated passage on media containing less and less blood, however, it may be acclimatized and will, in time, grow, although sparsely, upon ordinary nutrient agar. It does not, therefore, require the V and X factors that are essential to the development of

the hemophilic bacteria. The optimum temperature is 37° C., and the bacillus is aerobic and facultatively anaerobic.

H. pertussis is biochemically inactive. It does not form indol, does not reduce nitrates, and does not ferment any sugars. It is in part because of this inactivity and lack of the strict nutritive requirements that characterize the hemophilic bacteria that many prefer not to include it in the genus Hemophilus.

The resistance of *H. pertussis* to deleterious influences is feeble and of the same order as that of the influenza bacillus. It is killed by exposure to 55° C. for thirty minutes.

Toxins. As in the case of Pfeiffer's bacillus, the cell substance of *H. pertussis* is toxic upon parenteral injection into experimental animals and to about the same degree. Recent work seems to indicate definitely that an endotoxin is present in these bacilli which may be separated as a watery extract of disintegrated cells. There appear to be two fractions, one heat-stable and the other heat-labile and inactivated in thirty minutes at 56° C.[9] On intravenous inoculation in rabbits the heat-stable fraction produces a hyperglycemia and the heat-labile fraction a hypoglycem'a. Intratracheal inoculation in rabbits produces an edematous reaction followed by a lymphocytic infiltration about the blood vessels and bronchi which is reported to be similar to the changes produced in the lung in whooping cough. Extracellular toxin found in cultures of *H. pertussis* appears to be liberated endotox'n.

Variation and Antigenic Structure. Unlike the influenza bacillus, *H. pertussis* is generally in the smooth state when isolated from the body on an optimal medium, and is immunologically homogeneous. Leslie and Gardner[10] found that their newly isolated strains fell into four immunologic groups which they designated as Phases I, II, III and IV. Although not obviously rough, Phases III and IV are somewhat rougher in appearance and less stable in saline suspensions than the bacilli of Phase I. The S-R dissociation of *B. pertussis* occurs readily on culture on artificial media, even on blood agar, and it seems probable that these phases do not represent distinct immunological types but rather successive stages in the S-R transformation. The importance of the existence of the bacilli in Phase I for the preparation of vaccines and the like has been stressed by many workers in the last few years; it will be clear, however, that in all probability this is but another way of saying that the bacilli must be in the smooth state.

The S-R change, of which the above phases represent the early stages, proceeds to the obviously rough stage with consequent alterations in colonial morphology and loss of virulence.

As indicated elsewhere (p. 501), *H. pertussis* is immunologically related to *Brucella bronchiseptica*. Eldering and Kendrick[11] have described atypical bacilli isolated from a small proportion of cases of whooping cough which they designate *Bacillus para-pertussis*. These bacilli differ from *H. pertussis* in

[9] Evans and Maitland: Jour. Path. Bact., 1937, 45:715; Erich, Bondi, Mudd and Flosdorf: Amer. Jour. Med. Sci., 1942, 204:530; Evans: Jour. Path. Bact., 1943, 55:269; Sprunt and Martin: Amer. Jour. Path., 1943, 19:255.

[10] Leslie and Gardner: Jour. Hyg., 1931, 31:423.

[11] Eldering and Kendrick: Jour. Bact., 1938, 35:561.

that they grow readily on ordinary nutrient agar upon isolation and produce an alkalinity in litmus milk in two to four days. Immunologically, they are related to both *H. pertussis* and *Br. bronchiseptica.*

Pathogenicity for Man. Interest in *H. pertussis* has centered chiefly about the possible etiological role of this microorganism in whooping cough. Although this bacillus was early observed in the bronchial exudate, where it is present in enormous numbers in the early stages of the disease, failure to reproduce the disease in laboratory animals and other considerations led many to question the etiological significance of *H. pertussis.* For example, the presence of inclusion bodies (p. 842) observed by McCordock[12] has suggested a filterable virus etiology. At the present time, however, the chain of evidence formalized as Koch's postulates appears to be complete, and it may reasonably be concluded that *H. pertussis* is the causal agent of whooping cough.

The microorganism is constantly present in the disease, most frequently in the catarrhal stage which is known to be the most contagious, less frequently in the paroxysmal stage, and disappears during the decline, being rarely found after the fourth week of disease. It is not found in healthy persons who have not been in contact with whooping-cough patients, or in patients with infections of the upper respiratory tract other than whooping cough. By the cough-plate diagnostic method devised by Chievitz and Meyer[13] *H. pertussis* has been isolated in 88 per cent of the cases of whooping cough examined by Danish workers and in 98 per cent by Sauer and Hambrecht[14] in this country. The cellular infiltration and necrosis of the bronchi seen at autopsy in cases of whooping cough have been reproduced in the chick embryo by Gallavan and Goodpasture,[15] an interstitial pneumonia with leucocytic infiltration about the vessels and bronchioles and mucous secretion on the bronchial epithelium has been produced by intratracheal inoculation in the mouse[16] and in the rat by intranasal inoculation.[17] Rich[18] has produced symptoms similar to those of whooping cough in man by the inoculation of chimpanzees, and Mac-Donald and MacDonald[19] have recorded experimental pertussis in man.

Significant work upon the relation of *H. pertussis* to the characteristic manifestations of whooping cough has been carried out by Mallory and his co-workers.[20] The production of a mild toxin by the bacillus and the absorption of the toxin seem to be shown by the exudation of leucocytes into the lumen of the trachea and bronchi, by slight changes in the lymph nodules of the spleen, lymph nodes and gastro-intestinal tract, by the occurrence of the well-known lymphocytosis of whooping cough, and by the production of the antibody which makes possible the specific complement-fixation reaction. Possibly the cilia are damaged by a toxin, but this is not certain. More important than

[12] McCordock: Proc. Soc. Exp. Biol. Med., 1932, 29:1288.
[13] Chievitz and Meyer: Ann. Inst. Pasteur, 1916, 30:503.
[14] Sauer and Hambrecht: Jour. Amer. Med. Assn., 1930, 95:263.
[15] Gallavan and Goodpasture: Amer. Jour. Path., 1937, 13:927.
[16] Burnet and Timmins: Brit. Jour. Exp. Path., 1937, 18:83; Bradford: Amer. Jour. Path., 1938, 14:377.
[17] Hornibrook and Ashburn: Pub. Health Repts., 1939, 54:439.
[18] Rich: Bull. Johns Hopkins Hosp., 1932, 51:346; Rich et al.: Science, 1932, 76:330.
[19] MacDonald and MacDonald: Jour. Inf. Dis., 1933, 53:328.
[20] Mallory et al.: Jour. Med. Res., 1912–13, 22:115, 391.

toxic action seems to be the mechanical disturbance caused by the presence of the bacilli in the respiratory tract. By their presence in enormous numbers, dozens to a hundred or more between the cilia of a single cell (Fig. 103), they are thought to interfere seriously with the normal ciliary action. In consequence, the removal of secretions and inhaled particles is prevented, and the lungs are probably more exposed to infection by inhalation than under ordinary circumstances. The bronchopneumonia which sometimes develops in fatal cases of whooping cough may be due to *H. pertussis* or to other bacteria, such as the pneumococcus.

Bacteriological Diagnosis of Whooping Cough. The bacillus may be isolated by the cough plate method using the potato-glycerol-blood agar of Bordet and Gengou. The open plate is held 4 to 5 inches from the mouth and exposed to one or more explosive coughs. Characteristic colonies appear in two to three days but the plate should be retained for five days before discarding as negative. Nasopharyngeal swab cultures are reported to give a somewhat higher proportion of positive cultures than the cough-plate method.[21] The

Fig. 103. Whooping cough. Minute bacilli present in masses between cilia of two cells lining the trachea; × about 1500 (Mallory and Horner).

swab, on thin flexible copper wire, is passed through a nostril into the nasopharynx and left there for two or three coughs before withdrawal and culture on Bordet-Gengou medium. Sputum culture is not particularly satisfactory. Colonies of *H. pertussis* are larger and more opaque than those of the influenza bacillus; it may be differentiated further by hemolysis on blood agar and growth in the absence of the X and V factors.[22]

Epidemiology. An upper respiratory infection, whooping cough is transmitted by means of the secretions of the mouth and nose, to some extent through towels, handkerchiefs, hand-to-hand contact and the like, and undoubtedly to a great extent by droplet infection. Undiagnosed and atypical cases may play an important part in the dissemination of the disease. The age incidence is marked with 96 per cent of the deaths in children under five years of age. In 1945, for example, there were 1545 deaths from whooping cough in the registration area of the United States, a death rate almost five times that for the much more dreaded scarlet fever, and there is no doubt that whooping cough is one of the most important killing diseases of childhood. (Fig. 104.) There appears to be some racial difference in susceptibility since the Negro

[21] Saito, Miller and Leach: Amer. Jour. Pub. Health, 1942, 32:471; Miller, Leach, Saito and Humber: *ibid.*, 1943, 33:839.

[22] The laboratory diagnosis of whooping cough is discussed critically and in some detail by Donald: Brit. Med. Jour., 1938, Sept. 17, p. 613.

death rate is considerably higher than that of the whites. In contrast to most other diseases the incidence in females is somewhat higher than that in males. The seasonal incidence shows a drop during late summer and early fall, with
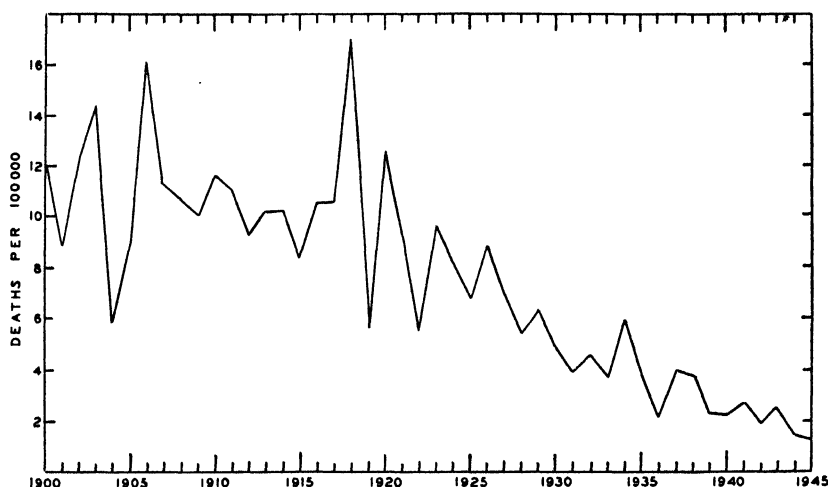
Fig. 104. The prevalence of whooping cough in the Registration Area since 1900 as indicated by the uncorrected death rates. From data compiled by the Bureau of the Census.

a prolonged peak in the later winter and spring (Fig. 105). The disease tends to recur in periodic epidemic waves, presumably a consequence of the accumulation of a new crop of susceptibles (Fig. 104).
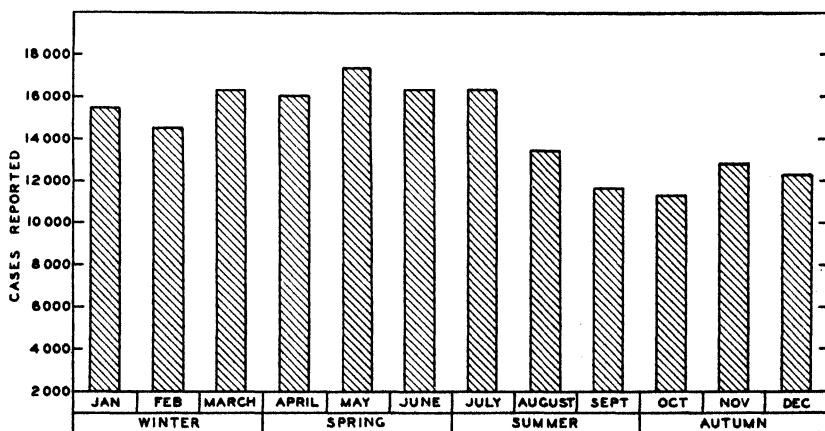
Fig. 105. The seasonal incidence of whooping cough. Averages of reported cases by months for the years 1939 to 1945 inclusive. Data from Supplements to Public Health Reports.

Immunity. Recovery from whooping cough is accompanied by the development of an immunity. Second attacks occur only infrequently in children and then are very mild; in older people second attacks are more severe. Com-

plement-fixing antibodies are formed but do not appear until the third or fourth week of the disease and, consequently, are of limited diagnostic value. It has been claimed that immunes give an allergic skin reaction to the intradermal injection of pertussis bacilli; the value of this reaction is uncertain.

Prophylactic Inoculation. The early attempts to use vaccines of *H. pertussis* were not uniformly successful. More encouraging results were obtained by Madsen and other Danish bacteriologists in the Faroe Islands. In the United States Sauer and his colleagues have prepared vaccines of freshly isolated, virulent strains which appear to be effective in reducing the incidence and severity of the disease. It appears to be of primary importance that the vaccine consist of the smooth bacilli of Phase I, and earlier reports of the inefficacy of active immunization are probably attributable in part, at least, to the use of other than the smooth form of the microorganism. Important parts of the immunizing antigen are soluble and removed by washing the cells; this material has been partially purified by methanol precipitation in the cold and studied by Pillemer, Burrell and Ross.[23] It is now definitely established that active immunization with properly prepared vaccine confers an effective immunity to pertussis.[24] Alum precipitated vaccine seems to be effective also but it is not known whether it is superior to plain vaccine. It appears also that pertussis vaccine may be successfully combined with diphtheria toxoid to allow immunization against the two diseases simultaneously; available evidence suggests that the immune response is equal to that obtained with separate antigens. It is generally agreed that the disease is appreciably milder in immunized children.

With the demonstration of the endotoxins of *H. pertussis*, considerable interest has attached to the possible role of anti-endotoxin in immunity to the disease. The toxicity appears to be neutralized with appropriately prepared antiserum and studies with experimental animals suggest that antitoxic antibody may be of importance in the immunity.[25] The results have not yet been applied to man except on a small scale.[26]

There is some evidence that antiserum may have prophylactic value[27] but neither antisera nor vaccines appear to have therapeutic value.

THE MORAX-AXENFELD DIPLOBACILLUS (HEMOPHILUS DUPLEX)

A small bacillus, described independently by Morax and by Axenfeld in 1896 and 1897, is responsible for infections of the conjunctiva and cornea in man, and is known as the Morax-Axenfeld bacillus or *Bacillus lacunatus* (from the lacunae of liquefaction produced by growth on coagulated serum). It has been grouped with the hemophilic bacteria as *Hemophilus duplex*.

The short rods, 1 μ by 2 to 3 μ, frequently appear end to end in pairs or

[23] Pillemer, Burrell and Ross: Science, 1947, *106*:36.
[24] See the general reviews by Felton and Willard: Jour. Amer. Med. Assn., 1944, *126*:294; Lewis: Med. Officer, 1946, 76:5; Parish, Gunn and Ungar: Pub. Health, 1946, 59:165.
[25] See Evans: Lancet, 1942, *i*:529; Anderson and North: Australian Jour. Exp. Biol. Med. Sci., 1943, *21*:1; Roberts and Ospeck: Jour. Inf. Dis., 1944, 74:14; Ospeck and Roberts: Jour. Inf. Dis., 1944, 74:22.
[26] Bullowa and Alterman: Jour. Amer. Med. Assn., 1942, *120*:886.
[27] *Cf.* Felton: Jour. Amer. Med. Assn., 1945, *128*:26.

short chains. They are non-motile, non-spore-forming and gram-negative. They do not grow on the ordinary nutrient media, potato, milk or gelatin but require the presence of serum, ascitic fluid or blood in the culture medium. They ferment few if any carbohydrates and do not form indol. Coagulated serum is liquefied. They die out within a day or two at room temperature but may survive for weeks in culture in the incubator. It has been reported[28] that hemolytic and non-hemolytic types occur which may be differentiated immunologically.

So far as is known this microorganism is pathogenic only for the human eye. The inoculation of experimental animals is without effect, but the instillation of the bacilli onto the conjunctival sac of man results in the development of a blepharoconjunctivitis, either chronic or acute, and severe inflammation of the cornea may be produced. Treatment with 0.25 per cent zinc sulfate solution is specific and produces a rapid cure, while silver salts are without effect. The
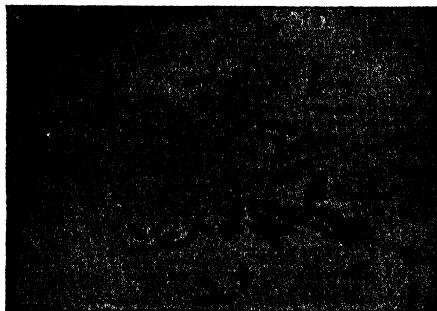
Fig. 106. Morax-Axenfeld diplobacillus; smear taken from conjunctiva (Brown Pusey).

disease is widely distributed and has been reported in Europe, Africa and North America.

DUCREY'S BACILLUS (HEMOPHILUS DUCREYI)

"Soft chancre" or "chancroid" is a venereal disease transmitted by direct contact. The lesions, which are on the genitals or adjacent areas, are irregular ulcers which differ from the hard or hunterian chancre—the primary lesion of syphilis—in that they are not indurated. Unlike syphilis, the infection remains localized, spreading no further than the neighboring lymphatics, which may become swollen to form secondary buboes in the groin.

The bacillus which bears his name was found by Ducrey in 1890 in the purulent discharge from the lesion, and by the inoculation of the skin of the forearm he was able to transmit the disease through fifteen generations. The microorganism was obtained in pure culture by Besançon, Griffon and le Sourd in the same year.

Ducrey's bacillus is a short rod 1 to 1.5 μ in length and 0.6 μ in breadth. In smears it is generally found to be ovoid, and there is a tendency to occur in end-to-end pairs or in short chains; in broth culture longer chains may be observed. It is non-spore-forming and nonmotile. The bacillus not infrequently

[28] Oag: Jour. Path. Bact., 1942, 54:128.

stains irregularly and bipolar staining may be observed. It is stained by the usual aniline dyes and is gram-negative.

The bacillus will not grow on the ordinary laboratory media and requires the addition of serum or, preferably, blood. According to Beeson[29] the X and V factors alone are not sufficient and additional factors present in either serum or erythrocytes are required. The small, grayish, glistening colonies appear in blood agar in twenty-four hours and, after two to three days' incubation, show a narrow zone of hemolysis. The bacilli may be cultivated from the chancroid by inoculating tubes of fresh (not more than three to five days old) rabbit blood. Smears made at the end of twenty-four to forty-eight hours' incubation will show the characteristic tangled chains of gram-negative bacilli.[30] These bacteria cannot be identified in smears from the chancroid because of a tendency to aberrant morphology.[31] The bacilli may be isolated and cultivated on the chorioallantois of the developing chick embryo, and appear to have little pathogenicity for the embryo.[32] Rabbits and monkeys may be infected by intradermal inoculation of pure cultures, but subcutaneous, intraperitoneal and intravenous inoculation is without effect.[33] Infected animals develop a hypersensitivity but no immunity to reinfection.

There is little or no immunity. The chancroid is frequently multiple and is auto-inoculable. A hypersensitivity develops, however, which is manifested as a reaction to the intradermal inoculation of killed bacilli, and which persists for many years. Antisera and autogenous vaccines are said to have therapeutic value.

[29] Beeson: Proc. Soc. Exp. Biol. Med., 1946, *61*:81.

[30] Beeson and Heyman: Amer. Jour. Syph., 1945, *29*:633.

[31] See the discussion of diagnosis by Greenwald: Jour. Amer. Med. Assn., 1943, *121*:9.

[32] Anderson and Snow: Amer. Jour. Path., 1940, *16*:269.

[33] See the discussion by Feiner, Mortara and Levenkron: Amer. Jour. Syph., 1945, *29*:71.

PSEUDOMONAS; LACTOBACILLUS; LISTERIA; BACTEROIDES; BARTONELLA; PLEUROPNEUMONIA GROUP; DONOVANIA

PSEUDOMONAS PYOCYANEA

The genus Pseudomonas includes some thirty species which are found, for the most part, in water, soil and wherever organic matter is decomposing. The fluorescent bacteria are members of this genus, *Ps. fluorescens* being the gelatin-liquefying form and *Ps. non-liquefaciens* the non-liquefying form. The elaboration of blue pigment by *Ps. syncyanea* results in "blue milk." One species, *Ps. septica*, is the cause of a disease of caterpillars, another, *Ps. jaegeri*, is pathogenic for chickens, and a third recently discovered species, *Ps. reptilovorus*, produces disease in certain reptiles.

The best known species of Pseudomonas, however, and the only one that is pathogenic for man, is *Ps. pyocyanea*. The blue or blue-green stains that sometimes appear upon surgical dressings long ago attracted attention, and even before the cause of the phenomenon had been discovered Fordos studied the pigment in 1860. Gessard found, in 1882, that the pigment was the product of a specific microorganism, *Ps. pyocyanea*, which he isolated in pure culture.

Morphology and Staining. The cells of *Ps. pyocyanea* vary considerably in size and proportion, but appear usually as small, slender rods, 1.5 to 3 μ long and 0.5 μ broad, frequently united in pairs and short chains. There are 1 to 3 polar flagella, and the bacterium is actively motile. Neither capsules nor spores are formed. The colonies are large and spreading, edges are irregular, and the consistency butyrous. These bacilli stain readily with the usual aniline dyes and are gram-negative.

Physiology. *Ps. pyocyanea* grows readily on all the ordinary culture media and most rapidly at a temperature of 30° to 37° C. Aerobic conditions are required, although it is sometimes said that there is some growth under anaerobic conditions. Gelatin is rapidly liquefied, hydrogen sulfide is produced, and indol formation is variable. The fermentative abilities of this bacterium are not great; acid is produced from dextrose, but most other carbohydrates are not attacked. An alkaline reaction is produced in litmus milk; a soft coagulum is formed, followed by rapid peptonization and reduction of the indicator.

One of the most distinctive characteristics of *Ps. pyocyanea* is its production of a bluish green, soluble pigment which does not color the colonies or other masses of growth but instead diffuses into the medium. There are actually two pigments formed. The one, *pyocyanin*, is a deep blue in color and can be extracted from aqueous solution by chloroform, and the other, a yellowish green

fluorescent pigment, is soluble in water but not in chloroform. They are both oxidation products of colorless precursors. Both sulfate and phosphate are required for the formation of pyocyanin. Pyocyanin is formed only by *Ps. pyocyanea*, but the fluorescent pigment is formed by several other species of Pseudomonas. These pigments may occur separately or together, and the conditions that determine their formation have been exhaustively studied.[1] Wrede[2] has determined the composition of pyocyanin, which has proved to be an entirely new type of dye and the first instance of a phenazine derivative occurring in nature (p. 124).

Pathogenicity. For some time after its discovery *Ps. pyocyanea* was generally regarded as a harmless saprophyte, or at the most as a microorganism of slight pathogenic power. It has since been learned that this bacterium is causally associated with a great variety of suppurative and other affections in
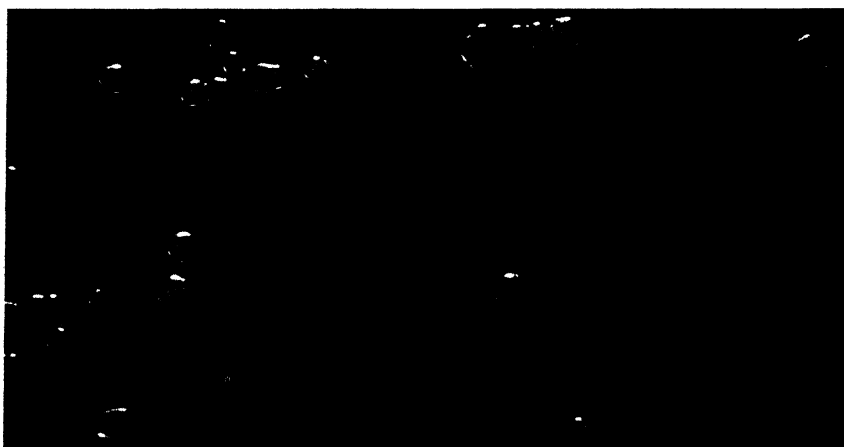
Fig. 107. Colonies of *Ps. fluorescens* on nutrient agar. Twenty-four-hour culture; × 3.

man. Apart from the many doubtful cases in which *Ps. pyocyanea* is found mixed with streptococci, staphylococci and other microorganisms where its share in inciting pathological processes is problematic, numerous instances are on record in which little or no question exists as to its etiological role. It has been found by a number of workers in pure culture in abscesses in different parts of the body, especially in the middle ear. Cases of endocarditis and pneumonia have also been met in which *Ps. pyocyanea* seemed to be the sole responsible microorganism. A generalized and fatal form of pyocyanic infection has been observed by a number of investigators, and the bacillus has been found in the blood during life. *Ps. pyocyanea* has been found constantly present in the intestinal discharges of patients during a dysentery-like epidemic, and the same microorganism was also present in abundance in drinking water which seemed, on epidemiological grounds, to be implicated in the outbreak. Ghosh[3] reported a series of cases of infection with this bacillus that closely

---

[1] Jordan: Jour. Exp. Med., 1899, 4:627.
[2] Wrede: Ztschr. f. Hyg. u. Infektionskr., 1930, *111*:90,
[3] Ghosh: Jour. Indian Med. Assn., 1938, 7:655

simulated Asiatic cholera. There is no doubt that under certain conditions *Ps. pyocyanea* is pathogenic for man, although probably human infections are relatively rare.

The intraperitoneal injection of 0.25 ml. of culture of a virulent strain will kill a guinea pig with acute symptoms in twenty-four hours. Smaller amounts are also fatal but less rapidly. Subcutaneous inoculation produces a marked local reaction. The symptom-complex presents nothing especially characteristic. Rabbits are not so susceptible as guinea pigs; mice and pigeons are less susceptible than rabbits. Immunity can be produced by small, non-lethal doses.
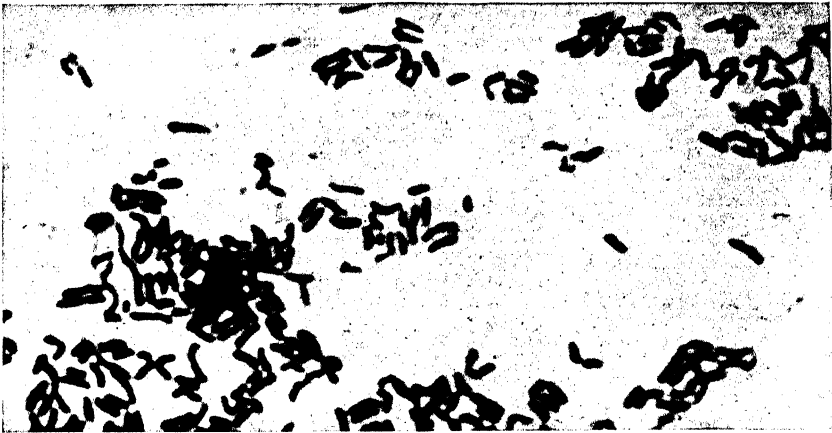
Fig. 108. *Lactobacillus sp.* isolated from the mouth. Morphologically identical with *L. acidophilus*. Note the diplobacillary form and palisade arrangement of the cells. × 2500 (Harrison).

The occurrence of *Ps. pyocyanea* as a plant pathogen and its close relationship, if not identity, with *Phytomonas polycolor*[4] has been noted elsewhere (p. 328).

LACTOBACILLUS

This genus is composed of a somewhat loose assemblage of microorganisms which produce considerable quantities of lactic acid from carbohydrates and which are able to withstand a degree of acidity usually fatal to non-sporulating bacteria. The latter characteristic is useful in the isolation of cultures as well as in distinguishing the group. Kendall has proposed a term, *aciduric* (acid-tolerant), which is now commonly applied.

Morphologically some of the lactobacilli are long, slender rods, while others are somewhat similar to the colon bacillus but unlike it they are all gram-positive. They are non-motile. Many cultures exhibit a typical diplobacillary form, often kidney-shaped. Old cultures frequently exhibit considerable pleomorphism. As a rule, cultures are facultatively anaerobic or microaerophilic, and some of them, particularly *L. bifidus*, are anaerobic on primary isolation.

Classification has been based primarily upon source of the cultures. Al-

4 Elrod and Braun: Jour. Bact., 1942, 44:633.

though this is an insecure criterion for differentiation, no really satisfactory basis of classification has been developed. A number of investigators have attempted classifications by means of carbohydrate-fermentation reactions. Many strains otherwise apparently identical differ, however, in fermentative reactions, and many cultures change in reaction after prolonged artificial cultivation. These variations make it doubtful whether such reactions are of any more value than morphological and colonial characters which are also subject to considerable variation. Efforts to classify the lactobacilli on the basis of metabolic activity have been more promising.

Bergey (1948) recognizes fifteen species, differentiated on a physiological basis, *viz.*,

(I) Homofermentative, *i.e.*, producing lactic acid primarily.
    (A) Optimum temperature of 37° to 60° C.
        (1) Acid from lactose
            (a) Optimum temperature 37° to 45° C.
                (i) Produce levo lactic acid
                      Lactobacillus caucasicus
                      Lactobacillus lactis
                (ii) Produce inactive or dextro lactic acid
                      Lactobacillus helveticus
                      Lactobacillus acidophilus
                      Lactobacillus bifidus
            (b) Optimum temperature 45° to 62° C.; usually maltose not fermented
                Lactobacillus bulgaricus
                Lactobacillus thermophilus
        (2) Lactose not fermented
            Lactobacillus delbrueckii
    (B) Optimum temperature 28° to 32° C.
        (1) Acid from lactose
            (a) Produce dextro lactic acid
                Lactobacillus casei
            (b) Produce inactive lactic acid
                Lactobacillus plantarum
        (2) Lactose not fermented
            Lactobacillus leichmannii
(II) Heterofermentative, *i.e.*, producing considerable amounts of products other than lactic acid, *viz.*, carbon dioxide, acetic acid, alcohol.
    (A) Optimum temperature 28° to 32° C.; usually ferment arabinose
        (1) Raffinose not fermented; sucrose and lactose usually not fermented
            Lactobacillus brevis
        (2) Ferment raffinose, sucrose and lactose
            Lactobacillus buchneri
            Lactobacillus pastorianus
    (B) Optimum temperature 35° to 40° C. or higher; arabinose usually not fermented
            Lactobacillus fermenti

The serological relationships of a number of species have been studied by several investigators. Harrison and co-workers,[5] using strains of oral origin, studied their polysaccharide precipitation reactions in an effort to establish an immunological classification. They reported the existence of four or more immunological types but indicated later that some strains are unstable, changes in

[5] Harrison *et al.*: Jour. Inf. Dis., 1939, 65:255; *ibid.*, 1942, 70:69; *ibid.*, 1942, 70:77; Jour. Dent. Res., 1944, 23:1.

immunological specificity occurring apparently in association with changes in fermentation reaction. Application of the agglutination reaction to the serology of the lactobacilli is hampered by a strong tendency to spontaneous agglutination, and earlier workers observed a marked serological heterogeneity. Williams,[5a] however, using filtered antigens and cross agglutinin absorption, has demonstrated the presence of four well-defined agglutinogens, designated A, B, C and D, whose distribution corresponds closely with the polysaccharide types of Harrison. There is evidence that cultures from the intestine include types identical with those obtained from the mouth.

Physiology. The lactobacilli are microaerophilic or anaerobic on primary isolation but after continued cultivation some strains will grow in the presence of air. Their nutritional requirements are complex and most strains cannot be cultivated on the usual nutrient or infusion media unless these are enriched by the addition of glucose or whey. These requirements have been studied in some detail and lactobacillus strains are frequently used for the microbiological assay of vitamins. For example, Dunn, Shankman and their co-workers,[6] in a study of the amino acid and vitamin requirements of twenty-three representative strains, found that individual requirements for amino acids varied from two to fifteen, and that pyridoxine, thiamine, riboflavin, biotin, folic acid, pantothenic acid and nicotinic acid are required in general, individual requirements, of course, differing.

Pathogenicity. Aside from a relation to dental caries (see below), the lactobacilli are generally regarded as non-pathogenic and are of interest largely in the dairy and fermentation industries. There are, however, reports in the literature of endocarditis caused by lactobacilli and febrile conditions apparently causally associated with a bacteremia.[7] These are perhaps a consequence of invasion from a dental lesion; a bacteremia may not be uncommon but pathological consequences seem to be very rare. The intestinal lactobacilli are a part of the normal flora as noted elsewhere and bear no relation to diarrheal disease.

Lactobacillus Acidophilus. First cultivated by Moro in 1900 from the feces of infants, this organism has been isolated from the intestine of nearly all the mammalia, many other vertebrates and some of the invertebrates. It increases in the intestine when the carbohydrate content of the diet is increased and may become predominant when a milk diet is administered.[8] These bacilli, fairly large and of variable length, are arranged singly, in pairs frequently slightly bent at the juncture, and in palisades (Fig. 108). Long chains, filamentous forms and club shapes are not uncommon. Young cultures stain uniformly gram-positive. Old cultures, however, often show beading or bipolar staining and may be easily destained so that they appear to be gram-negative. Colonies, usually small, may vary in shape between a smooth, rounded opaque form and a flattened, translucent, irregular form often having a ground-glass appearance. Fermentation reactions are variable,

[5a] Williams: Jour. Inf. Dis., 1948, 82:31.

[6] Dunn, Shankman et al.: Jour. Biol. Chem., 1947, 168:1, 23.

[7] Biocca and Seppilli: Arq. Biol. (São Paulo), 1944, 28:143; Jour. Inf. Dis., 1947, 81:112.

[8] Rettger and Cheplin: *A Treatise on the Transformation of the Intestinal Flora with Special Reference to the Implantation of Bacillus Acidophilus.* New Haven. 1933.

but most strains produce acid, no gas, from glucose, lactose, maltose and sucrose, and coagulate milk within forty-eight hours. The organism is used in the preparation of acidophilus milk, a buttermilk with considerable acidity, for which a number of therapeutic claims have been made.[9] Döderlein's bacillus (1892), (*Bacillus vaginalis, Bacillus crassus*), a common constituent of the flora of the vagina and believed to aid in the natural defenses against infection by contributing to the acidity of the vaginal secretions, is thought to be identical with *L. acidophilus.*

Lactobacillus Bifidus. Apparently closely related to *L. acidophilus* and often difficult to distinguish from it, *L. bifidus* is usually a thinner rod with ends somewhat more tapering and sometimes bifurcated (Fig. 109). It was isolated from feces of breast-fed infants by Tissier in 1900. Although common in the intestine of breast-fed infants, sometimes reaching over 90 per cent of the total intestinal flora, it is less conspicuous in the intestinal contents of bottle-fed babies. It is sometimes found also in the feces of

Fig. 109. *Lactobacillus bifidus.* Note the Y-shaped forms (Dack).

adult animals, including man. Antigenically it is closely related to *L. acidophilus* and like it produces acid, mainly lactic, from a number of sugars. Unlike the latter, it usually ferments inulin. It is anaerobic on primary isolation and some strains never grow well under aerobic conditions. Growth is enhanced by cystine. Partly because of its anaerobic requirements, it is sometimes classified with the Bacteroides. Tissier also isolated from feces of infants a similar organism (*Lactobacillus exilis*) which differs from the first in the possession of a more regular morphology and in greater facility of growth under aerobic conditions.

Lactobacillus Bulgaricus. This name is given to an organism isolated by Grigoroff in 1905 from Bulgarian fermented milk. It gained prominence through the work of Metchnikoff, who believed that intestinal putrefaction could be restrained by drinking milk fermented by this organism. When it was later shown that *L. bulgaricus* does not become implanted in the intestine, its use in experimental therapeusis was dropped in favor of *L. acidophilus.* More difficult to cultivate than *L. acidophilus*, slightly larger and somewhat different in sugar fermentations, it is nevertheless

[9] For an analysis of this subject see Rettger, Levy, Weinstein and Weiss: *Lactobacillus acidophilus and Its Therapeutic Application.* Yale University Press, New Haven. 1935.

closely related. Sherman and Hoge have reported[10] that *L. bulgaricus* rarely grows at 15° C., dies out on repeated culture in a lactose-peptone-yeast extract broth, is unable to grow in media containing 2.5 per cent NaCl and fails to grow in broth at *p*H 7.8, while *L. acidophilus* will grow under all these conditions. The Boas-Oppler bacillus, first seen in 1895 in the gastric juice of patients suffering from carcinoma of the stomach and cultivated
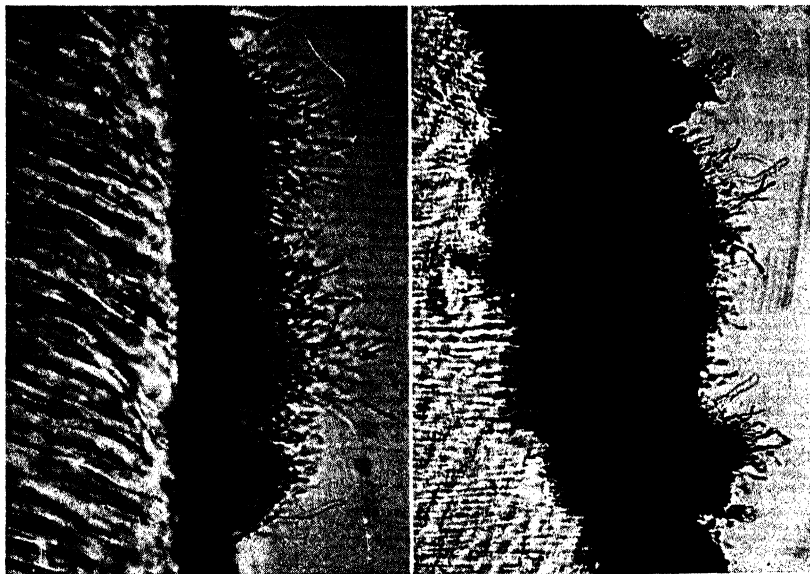
Fig. 110.   Fig. 111.

Fig. 110. Ground section of human tooth enamel showing an unstained bacterial plaque growing on the surface. Note the enamel rods and filamentous bacteria. × 1200 (Blayney).

Fig. 111. Ground section through an early carious lesion in human tooth enamel stained with gentian violet. Note the intimate contact of the bacterial plaque with the eroding surface. × 500 (Blayney).

by Heinemann and Ecker[11] from patients with this and other gastric diseases, is a member of this group, similar to, if not identical with *L. bulgaricus*.

DENTAL CARIES

There is much disagreement concerning the basic factors responsible for susceptibility and resistance to dental decay, and there is a wide diversity of opinion as to the relative importance of general systemic conditions, diet, bacterial flora of the mouth and various local factors in the oral environment. Nevertheless, it has been well established that certain microorganisms, particularly members of the lactobacillus genus, are usually associated with the disease and, although there is not as yet direct experimental proof, considerable indirect evidence has been adduced to indicate that this association is

[10] Sherman and Hoge: Jour. Bact., 1940, 40:11.
[11] Heinemann and Ecker: Jour. Bact., 1916, 1:435.

etiological.[12] It has not been shown, however, that other carbohydrate-fermenting, acid-producing organisms are not implicated also.

Much of the evidence that lactobacilli are associated with dental caries has been obtained from cultures of saliva. Many investigators have found close correlation between the numbers of lactobacilli in saliva and the degree of carious involvement of the teeth. Such a correlation is by no means invariable, however. In some studies lactobacilli have been found to be a minor constituent of the salivary flora and to be infrequent in or absent from the flora of carious cavities. Bradel and Blayney[13] in a study of some 4000 saliva samples from carious patients found in the majority of cases a fair degree of correlation between the numbers of lactobacilli and the amount of tooth decay. There were, however, more than 900 samples in which they did not find any lactobacilli. Bibby[14] has suggested that the association of lactobacilli with dental caries is a result of the favorable habitat furnished by the cavities in the teeth. A number of dental bacteriologists differ from this view and interpret the results of saliva cultures as indicating a correlation with the degree of caries "activity" rather than with the amount of cavitation present. A "caries activity test" based on this hypothesis has been developed.

Most of the present-day theories of the etiology of dental caries, whether they assign primary or secondary roles to the oral flora, are based on the hypothesis that the actual process of decalcification is due to the action of organic acids elaborated on the tooth by microorganisms. Areas of the tooth surface which are protected from the cleansing action of roughage in the diet are covered by bacterial plaques, thin, tenacious, felt-like layers composed of leptotrichia, actinomyces and a variety of other microorganisms including chiefly bacillary and coccal forms. A section of a tooth with a plaque attached is shown in Fig. 110. The underlying enamel is sound.

A relationship of the bacterial plaque to the initial stages of dental caries was first clearly postulated by Black[15] near the turn of the century but little experimental confirmation has been advanced until recently. After caries begins under a plaque it extends through the enamel as in the illustration in Fig. 111, and the dentin becomes involved. The lesion in the dentin extends laterally at the junction of enamel and dentin, and centrally as bacteria invade the dentinal tubules (Fig. 112). It eventually reaches the pulp, or so-called "nerve," and the tissues around the root may finally become infected.

While acid production by the microorganisms on the tooth is no doubt controlled to a considerable extent by the amount and nature of the food retained on the surface, dental caries does not occur in all food-retentive areas. Localization of the initial lesion, dependent partly perhaps upon a variety of local factors, is apparently determined primarily by the nature of the plaque. Although knowledge of the bacterial flora of the plaque is

[12] For reviews see Ch. VIII by T. Rosebury and Ch. X by P. Jay in *Dental Science and Dental Art*, edited by S. M. Gordon. Lea & Febiger, Philadelphia. 1938.

[13] Bradel and Blayney: Jour. Amer. Dent. Assn., 1940, 27:1601.

[14] Bibby: In *Dental Caries*. University of Pennsylvania Press, Philadelphia, 1941, pp. 27–43.

[15] Black: *Operative Dentistry*. Medico-Dental Publishing Co., Chicago, 1908, Vol. 1, p. 75.

still incomplete, recent work indicates that certain types of lactobacilli, vigorous in acid production and highly acid-tolerant, are commonly present in plaques covering areas of beginning caries and are found infrequently in those removed from sound enamel surfaces. Stephan[16] has found the reaction of plaques over caries-active areas to be *p*H 5.0 or less immediately following a glucose rinse. A smear from a typical carious plaque showing lactobacilli as well as a number of other forms of microorganisms is illustrated in Fig. 113. Although the basic bacterial flora of the plaque may vary considerably, the filamentous forms are apparently always present. Whether
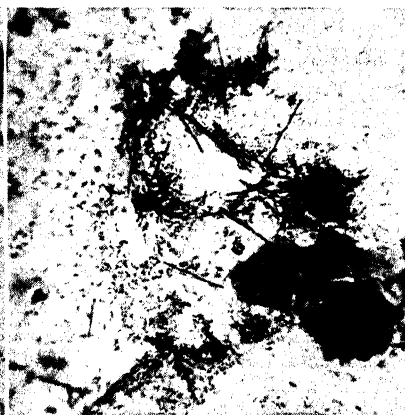
Fig. 112.   Fig. 113.

Fig. 112. Ground section of carious human dentin showing bacteria in the dentinal tubules. × 1000 (Blayney).

Fig. 113. Smear of material from a fragment of a bacterial plaque like that illustrated in Fig. 106. Note the bacillary forms which have been teased out of the filamentous masses. × 1000 (Blayney).

they play any role in the caries process other than that of entrapment and protection of other bacteria is not known.

RELATED BACTERIA

Microbacterium. Forms which are closely related to the lactobacilli and produce lactic acid without gas in carbohydrate fermentation, but which are aerobic, are grouped in the genus Microbacterium. Two species are recognized, *Microbacterium lacticum* and *Microbacterium flavum*. The first occurs in the intestinal tract, and the second predominately in dairy products. Their relatively high heat resistance is of significance in the latter.

Propionic Acid Bacteria. A group of bacteria closely related to the lactobacilli but characterized by the production of propionic acid and acetic acid in the fermentation of carbohydrates and polyhydric alcohols is known as the propionic acid bacteria. The original single species, *Bacterium acidi propionici*, has been split into a number of species on a physiological basis under the generic name Propionibacterium. Of the eleven species recognized by Bergey (1948) all but one are anaerobic or microaerophilic.

16 Stephan: Jour. Dent. Res., 1944, 23:257.

They are non-motile, non-spore-forming, gram-positive bacilli somewhat resembling the diphtheroids in microscopic morphology in that they contain metachromatic granules and the cells are often club-shaped and show branching. Growth is relatively slow, and their nutritional requirements are complex in that yeast extract media supplemented with lactate or monosaccharides are required to support growth. These bacteria are of some industrial importance and have been widely used in studies on the mechanisms of bacterial fermentation of sugars. They seem to be completely non-pathogenic.

LISTERIA

A small gram-positive bacillus was isolated in 1926 by Murray, Webb and Swann[17] from rabbits having a disease characterized chiefly by a large mononuclear leucocytosis. It was originally named *Bacterium monocyto-*
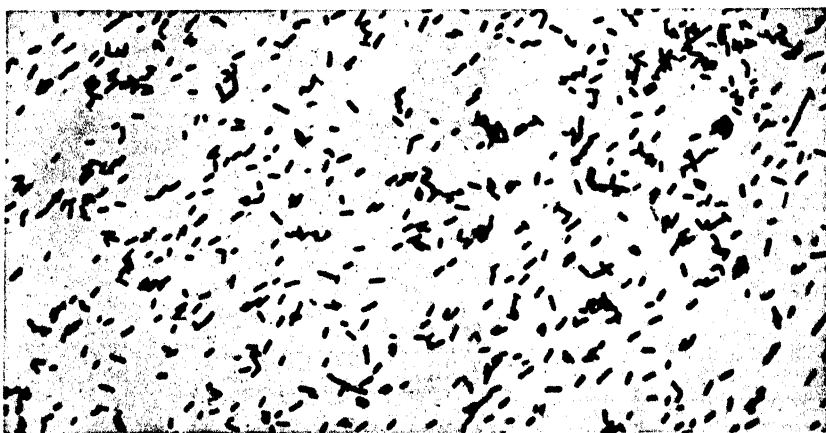
Fig. 114. *Listeria monocytogenes.* Smear from a pure culture. Fuchsin; × 1050.

genes, but is now generally known as *Listeria monocytogenes* and infection with this bacterium is called *listerosis.*

The microorganism is a small rod motile by means of a single polar flagellum and 0.5 μ in breadth and 1 to 2 μ in length. Neither spores nor capsules have been observed. The cells occur singly, in V-shaped or parallel pairs, and in short chains. They usually stain uniformly with the ordinary aniline dyes, though sometimes the stained cells appear beaded. Colonies on solid media are small, transparent by transmitted light, and milk-white by reflected light. They are hemolytic on blood agar, and appear not unlike hemolytic streptococci or some of the diphtheroids.

Growth is best on enriched media such as liver extract and blood agar. The optimum temperature is 37° C. This bacterium is aerobic and facultatively anaerobic. It is relatively inactive biochemically. Acid but no gas is produced promptly from dextrose, rhamnose and salicin; the fermentation of sucrose, maltose, lactose, glycerol and starch is irregular and slow. Arabinose, galactose, xylose, mannitol, dulcitol, inulin and inositol are not

[17] Murray, Webb and Swann: Jour. Path. Bact., 1926, 29:407.

fermented. Gelatin is not liquefied, indol is not produced, and nitrates are not reduced to nitrites.[18]

Julianelle and Pons[19] found that a large group of representative strains fell into two serologic types by agglutination and precipitin tests. One of these was associated with rodent infections and one with ruminant infections.

Pathogenicity for Animals. Natural infection of animals has been observed with some frequency and appears to be of greatest importance in ruminants in which it is usually associated with encephalitis or encephalomyelitis. Encephalitis likewise commonly occurs in infected swine, while a generalized infection is usually found in rodents and fowls.[20] The mouse, guinea pig, rabbit and rhesus monkey may be infected experimentally, the mouse being the most susceptible. Strains of the bacterium vary considerably in virulence, the most virulent producing a fatal infection in mice and guinea pigs by intraperitoneal inoculation of 10^{-6} ml. of twenty-four-hour broth culture. In the rabbit a marked increase in circulating monocytes is often produced and at autopsy multiple necrotic foci in the liver, meningeal inflammation and myocardial abscesses are found. Julianelle has described a characteristic conjunctivitis and keratitis that may be produced in the eye of the rabbit by instillation or swabbing and which serves to differentiate this bacterium.

Pathogenicity for Man.[21] This bacterium is of undoubted pathogenicity as indicated by reports of a number of cases of Listeria meningitis in which the case fatality rate has been about 70 per cent. Listerosis in man does not appear to be common, however; about twenty cases have been reported in all. It is presumed that the infection is acquired from lower animals but there is no proof of this. Infection in man has been of particular interest in connection with the etiology of infectious mononucleosis.

Infectious Mononucleosis (Glandular Fever, Pfeiffer's Disease). An acute, rarely fatal disease of man known as infectious mononucleosis or glandular fever is characterized by fever, sore throat, enlargement of the lymphatics and spleen, and a lymphocytosis. Heterophile antibody is found and the agglutination of sheep red cells, the Paul-Bunnell test, is diagnostic in the nonspecific sense that it is associated with the disease.[22] *Listeria monocytogenes* has been isolated from a number of cases of the disease and this, coupled with the ability of the bacterium to produce a monocytic leucocytosis in experimental animals, has led a number of workers to suspect an etiological relationship. Too few cases have been studied to establish this; such evidence as there is has been critically reviewed by Webb.[23]

---

[18] For detailed studies see the review by Julianelle: Jour. Bact., 1941, 42:367; and the studies of Harvey and Faber: Jour. Bact., 1941, 42:677.

[19] Julianelle and Pons: Proc. Soc. Exp. Biol. Med., 1939, 40:364; also Drew: *ibid.*, 1946, 61:30.

[20] See the review by Graham, Levine and Morrill: Bull. Illinois Agr. Exp. Station. No. 499, 1943.

[21] *Cf.* Julianelle: Ann. Int. Med., 1940, 14:608.

[22] For a survey of the literature on this disease see Smeall: Edinburgh Med. Jour., 1942, 49:291.

[23] Webb: Lancet, 1943, ii:5.

19

NON-SPORE-FORMING ANAEROBIC BACILLI (BACTEROIDES)

There is a large group of non-spore-forming anaerobic bacilli that are usually gram-negative. Normal inhabitants of the upper respiratory tract, genital tract and colon, where they may outnumber the aerobic flora, the microorganisms are not infrequently associated with ulcerative processes of the mucous membranes and may, under appropriate circumstances, invade the tissues and organs of the body with the production of abscesses, or the blood stream to give rise to septicemia. Generally neglected in routine bacteriological examinations, these bacteria may be present in "sterile pus" from surgically drained abscesses and similar affections where bacteria are not found by the usual cultural methods. Dack[24] has noted their presence in 200 of 5180 specimens from the Department of Surgery of the University

Fig. 115. *Bacteroides funduliformis.* The swollen and filamentous forms and poorly staining "ghost cells" are typical of the usual stained smear preparations. × 1000 (Dack).

of Chicago submitted for routine bacteriological examination, an incidence of about 4 per cent.

The relation of these microorganisms to other bacteria is uncertain. As a group they probably make up several genera and cannot be regarded as species of a single genus except tentatively. They are morphologically heterogeneous, varying from slender rod forms which may be tapered toward the ends, the so-called fusiform bacilli, to filamentous and branching forms characteristic of the higher fungi. The usual appearance of stained smear preparations is illustrated in Fig. 115 and details of the cell morphology in the electron micrographs in Fig. 116. The high degree of pleomorphism characteristic of these forms has been shown by Smith, Mudd and Hillier[25] to be due in large part to a mode of reproduction in which large round bodies are formed from which daughter cells separate. These usually resemble the bacillary parent cells but sometimes occur as much smaller elements in the so-called L type of variation. The process by which this occurs is illustrated in the series of electron micrographs in Fig. 117 which were arranged for present purposes by Dr. Smith from Smith, Mudd and Hillier.[25]

[24] Dack: Bact. Rev., 1940, 4:227.
[25] Smith, Mudd and Hillier: Jour. Bact., 1948, 56:603.

Single species have been given a variety of generic names, including Bacillus, Bacterium, Necrobacillus, Bacteroides, Corynebacterium, Streptothrix, and Actinomyces. Descriptions of these bacteria have been compiled by Weinberg, Nativelle and Prevot,[26] and Prevot[27] has suggested a more or less elaborate classification. In the absence of further information neither the relation of these anaerobic forms to other bacteria nor their relation to one another can be satisfactorily formulated, and they will be discussed here under the single generic name *Bacteroides*.

Those forms which are present in great numbers in normal feces will grow upon the usual laboratory media. Those which are associated with

Fig. 116. *Bacteroides funduliformis.* Electron micrographs. Right, a pair of cells resulting from simple fission, fixed in formalin; × 3300. Left, swollen cells containing granular material, especially in the swollen areas, fixed in formalin; × 4900. (Smith, Mudd and Hillier, Jour. Bact., 1948, 56:603.

pathological processes in man, however, are nutritively fastidious and require infusion media enriched by the addition of blood, yeast or vegetable extracts and similar substances, together with glucose and cysteine. In some cases they may be isolated on beef or veal infusion blood agar. Some strains grow in amino acid media supplemented with all the known bacterial vitamins; pyruvic acid appears to be of considerable importance in the nutrition of these organisms. The optimum pH is 6.3 to 7.0 and the optimum temperature for growth is 37° C. Completely anaerobic conditions are essential and growth is favored by the presence of carbon dioxide.

The better known of these bacteria may be described briefly.

Bacteroides Fusiformis (*Bacillus fusiformis, Fusiformis fusiformis, Fusobacterium plauti-vincenti*). These fusiform bacilli are found in ulcerative

[26] Weinberg, Nativelle and Prevot: *Les Microbes Anaerobies.* Masson et Cie., Paris. 1937.

[27] Prevot: *Manuel de Classification et de Determination des Bacteries Anaerobies.* Monographies de l'Institut Pasteur. Masson et Cie., Paris. 1940.

stomatitis (trench mouth) and Vincent's angina. Their relation to the spiro-chetes with which they are usually associated is considered elsewhere (p. 735). These forms are regarded by Bergey as the type species of the genus Fusobacterium. They appear as slender rectilinear or incurving bacilli and frequently assume a filamentous form. They are gram-negative and tend to stain irregularly with the ordinary aniline dyes. Acid but no gas is pro-duced from dextrose, levulose, sucrose, maltose, and sometimes from lac-tose. They may be isolated on blood agar incubated anaerobically, and the colonies are small and surrounded by a zone of green hemolysis. *Bacteroides fusiformis* is not pathogenic for experimental animals in pure culture, but in mixed culture produces abscesses.

Bacteroides Fragilis (*Bacillus fragilis, Fusiformis fragilis*). This bac-terium was found by Veillon and Zuber in twenty-two cases of appendicitis and since has been found in lung, pelvic and hepatic abscesses, in septicemias with metastatic abscesses, and in infections of the urinary tract. The cells are small, slender rods, sometimes slightly curved. They are gram-negative and non-motile. *Bacteroides fragilis* is difficult to isolate but will grow on the usual laboratory media. The colonies are small (less than 1 mm.) and trans-parent. No hemolysis is apparent on blood agar. There is a marked tendency to autolysis with apparent resorption of the colonies and broth cultures are no longer viable after seven to eight days' incubation. A variety of sugars are fermented to acid and gas. The pathogenicity of this bacterium for experi-mental animals is uncertain.

Bacteroides Funduliformis (*Actinomyces necrophorus, Fusiformis necrophorus, Streptothrix necrophorus, Bacillus necrophorus, Corynebac-terium necrophorum,* Schmorl's bacillus). These bacilli have been found in abscesses of the liver, lung and other parts of the body, in chronic ulcerative colitis and in the blood stream. Infections with these microorganisms are probably more common than is generally realized. *Bacteroides funduliformis* has also been found in lower animals as in bovine liver abscesses. The bacilli are highly pleomorphic; slender straight and curved rods may be found inter-mingled with filamentous and swollen forms, and "ghost cells" which do not stain are frequent (Fig. 115). There is a marked tendency to irregular stain-ing and the bacilli are gram-negative. The colonies on blood agar are variable in size from plate to plate and surrounded by a zone of green hemolysis which may become clear upon prolonged exposure to the air. Glucose, mal-tose and levulose are fermented to acid, and there is no evidence of proteo-lytic activity in gelatin or coagulated egg-white cultures. Some strains give rise to a spreading necrotic lesion upon subcutaneous injection in the rabbit which is usually fatal, while others produce only a localized lesion. Guinea pigs are relatively resistant.

Bacteroides Ramosus (*Fusiformis ramosus, Ramibacterium ramosum, Bacillus ramosus.* Not to be confused with *Bacillus ramosus,* a name some-times applied to *Bacillus mycoides*). These bacteria are frequently present in the pus of appendicitis and have been encountered in pulmonary gan-grene.[28] They appear as small, slender rods which often show branching Y forms and pseudofilaments. They are gram-positive. *Bacteroides ramosum*

[28] *Cf.* Jour. Amer. Med. Assn., 1937, *108*:1902.
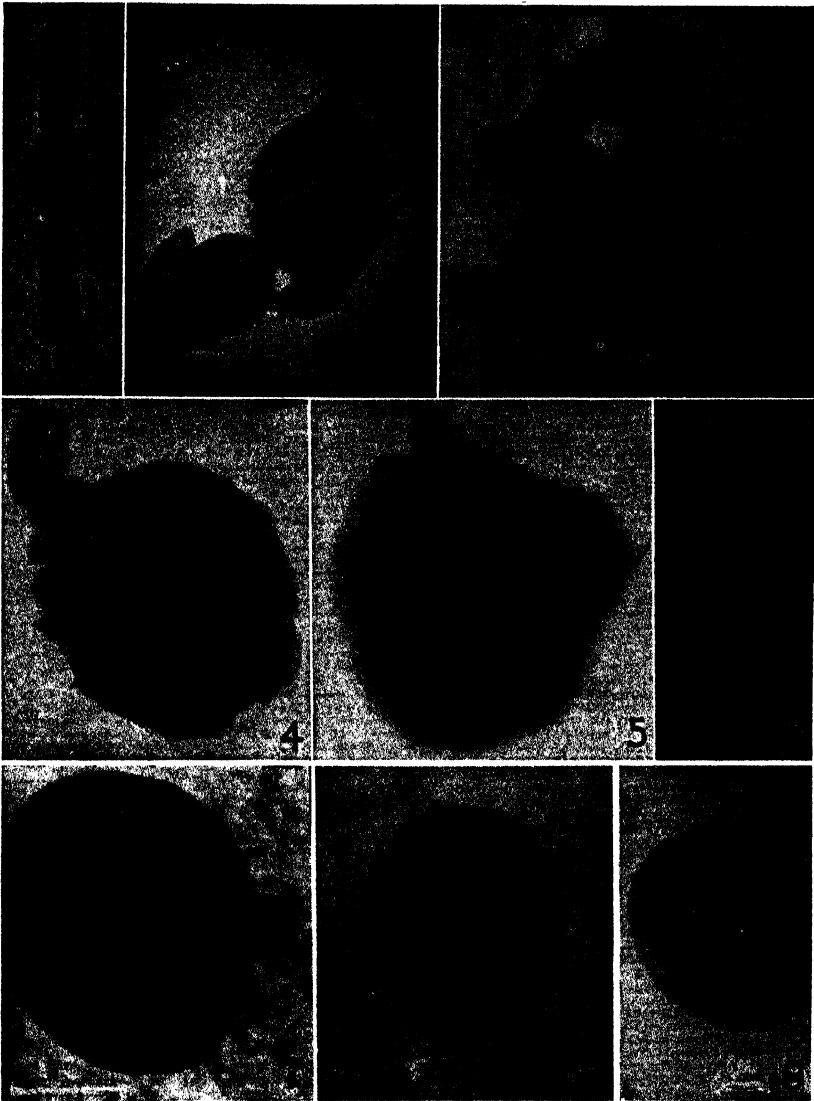
Fig. 117. Reproduction by large bodies in *Bacteroides funduliformis*. In liquid media the cells develop rounded enlargements (1) which increase in size (2) to yield large round bodies (4–9). These germinate by extrusion of multiple filaments (3). Note that the filaments are sheathed with the cell wall of the large body. The intracellular dark material pinches off within the filaments, which then segment to yield the new daughter cells. The large round bodies contain a dark material that is sometimes diffuse (4 and 5), and sometimes present in discrete masses (6) or thread-like filaments (7). In 8 and 9 it appears to lie in a single large mass. Part 7, × 10,600, and remainder, × 4500. (Rearranged from Smith, Mudd and Hillier by Dr. Smith.)

is pathogenic for experimental animals, giving rise to abscesses in rabbits and guinea pigs on subcutaneous injection; fatal infections are produced by intravenous inoculation.

Bacteroides Melaninogenicus (*Ristella melaninogenica*). This micro-organism has been found in the mouth, tonsils, infected abdominal wounds, focal infections of the kidneys, in stools from patients with chronic amebic dysentery, and in puerperal sepsis. It is described by Burdon[29] as a very small gram-negative diplobacillus. On blood agar its colonies are black owing to the slow (four to five days) formation of a melanin-like pigment. *Bact. melaninogenicus* grows well in mixed culture, but sparsely in pure culture. It is difficult to obtain in pure culture and, when admixed with other bacteria in a colony, colors the entire colony black. Acid is formed from dextrose, levulose, lactose, maltose, sucrose and mannitol. It is markedly proteolytic and rapidly digests coagulated serum and other native proteins. Its pathogenicity as a primary invader is uncertain.

Bacteroides Pneumosintes (*Dialister pneumosintes*). *Bacteroides pneumosintes* was described by Olitsky and Gates[30] as the causal agent of influenza. Now known to bear no relation to this disease, its pathogenicity for man is uncertain. It may be cultured from nasopharyngeal washings in Smith-Noguchi medium (human ascitic fluid containing a piece of sterile rabbit kidney, and covered with a petrolatum seal) and after a few transfers will grow anaerobically on blood agar, chocolate agar and Bodet's medium. These bacteria are minute bodies arranged singly, in pairs, or in short chains; they are non-motile and gram-negative. Because of their small size they pass Berkefeld V and N filters. They produce acid but no gas from dextrose; other sugars are not fermented. If mass cultures are injected intra-tracheally into rabbits there is a rise in temperature and sometimes a con-junctivitis and mononuclear leucopenia with recovery in two or three days. If the animal is sacrificed the lungs are found to be edematous with hemor-rhages on the surfaces. *Bact. pneumosintes* is not pathogenic for monkeys.

BARTONELLA

Bartonella Bacilliformis. Oroya fever, an infectious anemia, and ver-ruga peruana, a disease characterized by miliary or nodular eruptions, have existed for centuries in certain districts in Peru, and recently have been found in Colombia and Ecuador. It was shown by Carrion, through fatal self-inoculation, that the two are stages or manifestations of a single disease which is now commonly known as *Carrion's disease*. The etiologic agent is a small pleomorphic bacillus which was observed by Barton in 1905 and named *Bartonella bacilliformis* by Strong, Tyzzer and Sellards.[31]

Morphology and Staining. Bartonella bacilliformis is a small, motile, aerobic, gram-negative bacillus 0.2 to 0.5 μ in diameter and 1 to 2 μ in length which is found as a slightly curved rod occurring singly, end to end in pairs, and in short chains. A rounded ovoid form, 0.3 to 1 μ in diameter,

[29] Burdon: Jour. Inf. Dis., 1928, 42:161.

[30] Olitsky and Gates: Jour. Exp. Med., 1921, 33:125, 361, 713; *ibid.*, 1922, 35:813; *ibid.*, 1922, 36:501.

[31] Strong, Tyzzer and Sellards: Jour. Amer. Med. Assn., 1915, 64:806.

is also observed singly, in pairs and in groups. It stains reddish violet with Giemsa's stain, sometimes showing a reddish purple granule at one end of a bluish rod.

Physiology. This microorganism was first cultivated in semisolid leptospira medium and later in tissue culture and in the developing chick embryo, though it could not be carried in serial transfer in the last. Sparse growth occurs on cystine-dextrose-blood agar and apparently the X factor is required while the V factor is not. Gieman[32] has developed a tryptone-serum medium in both liquid and solid form which supports excellent growth. Little is known of its physiological processes.

Pathogenicity for Man. As indicated above, the disease occurs in two forms, the systemic form in which the red cells are infected, and the histoid cutaneous
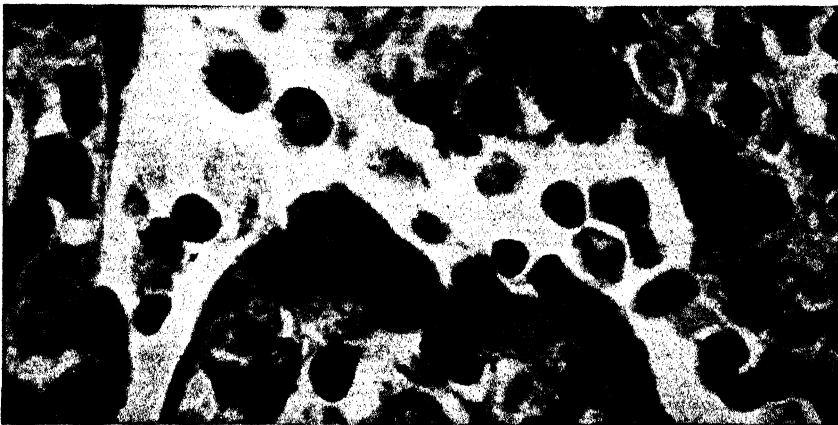
Fig. 118. *Bartonella bacilliformis* in human spleen. Note the huge numbers of the micro-organisms packed into the lining cells. Giemsa; × 1450 (Humphreys).

form. The two may exist independently, coexist or occur separately, though the usual course is the systemic form followed by the cutaneous form if the former is not fatal, or the cutaneous form alone. The first is a severe, often fatal, febrile anemia. The incubation period is given as about three weeks. The anemia is frequently severe, and the loss of red cells may be 200,000 to 300,-000 per cubic millimeter per day until the total count is half a million or less. The anemia is thought to be due to the direct action of the microorganisms on the erythrocytes, including hemolysis, and to tissue hemorrhages. The fever is of an irregular remittent type, and pain in the bones, joints and head is common. The case fatality rate is 20 to 40 per cent, death occurring in two to three weeks after onset. With recovery the eruptive stage of the disease appears and persists for two to three months. Whether it follows the systemic form or is the primary clinical manifestation of infection, this stage is characterized by a miliary or nodular eruption; the former is by far the more common. The miliary eruption is most common on the face and extremities, appearing as a macule which becomes nodular and eventually disappears leaving no scar. The nodular type of eruption develops more slowly, the nodules may become 2 to

[32] Gieman: Proc. Soc. Exp. Biol. Med., 1941, 47:329.

3 cm. in diameter and have a tendency to become strangulated. They are formed by the proliferation of the endothelial cells of the vessels which become obstructed with an inflammatory exudate of plasma cells and fibroblasts, and show a marked tendency to hemorrhage.

Bartonella cells are found in large numbers within the erythrocytes in Oroya fever and may be demonstrated in Giemsa-stained blood smears. In both forms of the disease they are found in the tissue macrophages, especially the vascular endothelial cells of the lymphatics, spleen and liver, often in large clusters within individual cells. It is reported that blood cultures are frequently positive in the systemic infection but it is not clear whether this is a reliable method of diagnosis.

Epidemiology. Bartonellosis is strictly limited geographically, being, so far as is known, exclusively American and tropical, occurring in the Andes between latitudes 2° N. and 13° S. It is transmitted by *Phlebotomus verrucarum* and *P. noguchii* in Peru; whether other species of Phlebotomus or other arthropods, such as Dermacentor, are natural vectors is not known. The reservoir of infection, other than asymptomatic infections and clinical disease in man, is not known but domestic animals, including chickens, and guinea pigs and field mice are suspected.[33]

Immunity. It is generally said that recovery from an attack of either form of the disease confers a solid immunity to both. With the cultivation of *B. bacilliformis,* it has been possible to study the occurrence of circulating antibody, agglutinins, and investigate the possibilities of prophylactic inoculation. Howe[34] has shown that agglutinins may be demonstrated during the early stages of the disease but, in spite of the lasting immunity, almost always disappear with the subsidence of clinical symptoms. Their diagnostic significance is not as yet known. Howe and Hertig[35] have carried out a limited series of inoculations with formalized suspensions of *B. bacilliformis* with encouraging results.

Pathogenicity for Animals. In general experimental animals appear to be highly resistant to infection with *B. bacilliformis.* Both the eruptive and systemic forms of the disease have been produced in rhesus monkeys, the latter in splenectomized animals.

Bartonellosis of Animals. Naturally occurring bartonellosis has been found in a number of animals, including the dog, cattle and a variety of rodents. The infection takes a systemic rather than eruptive form and is usually latent but activated when host resistance is reduced as by splenectomy. The bartonella-like organisms of lower animals have been separated into three genera, Haemobartonella, Grahamella containing but a single species (*Grahamella talpae*) which differs from Haemobartonella in that the infection is not eradicated by treatment with arsenicals, and Eperythrozoon, which is more highly pleomorphic than the first two groups and is found in the plasma as well as in the erythrocytes. None of these forms have been cultivated and all appear to be non-pathogenic for man.

[33] For an excellent review of the present status of human bartonellosis see Mera: Bol. Oficina Sanitaria Panamericana, 1943, 22:304.

[34] Howe: Jour. Exp. Med., 1942, 75:65; Arch. Int. Med., 1943, 72:147.

[35] Howe and Hertig: Jour. Immunol., 1943, 47:471.

Of these, the most commonly encountered is *Haemobartonella muris* which infects rats and is transmitted by the rat louse, *Haematopinus*, and the rat flea, *Xenopsylla cheopsis*. The infection is very common and the great majority of laboratory rats are infected. The infection is latent, and may be precipitated by splenectomy in an acute form with the appearance of parasitized red cells in the circulating blood. Special strains of rats are maintained bartonella-free for experimental purposes. *Haemobartonella canis* is the cause of an infectious anemia of dogs and is transmitted by the dog flea, *Ctenocephalus*. *Haemobartonella tyzzeri* occurs in guinea pigs, *Haemobartonella microtii* in the vole, *Haemobartonella bovis* in cattle, and other species in mice, shrews and squirrels.

Eperythrozoönosis similarly occurs as a latent infection which is activated by splenectomy. *Eperythrozoon coccoides* is a parasite of white mice and other species occur in wild mice. The disease also occurs in sheep and cattle and the causative organisms are *Eperythrozoon ovis* and *Eperythrozoon wenyonii* respectively.

The Systematic Position of Bartonella. The relationship of these microorganisms to the bacteria on the one hand and to the rickettsiae and viruses on the other is of some interest. Among the bacteria the tendency to parasitize the cells of the host and appear as intracellular clumps of microorganisms is most marked in *Pasteurella tularensis* which commonly is found in an intracellular position. Bartonella shows a much more marked preference for intracellular parasitism and both in this respect and in morphology seems to be closed related to the rickettsiae. It is generally agreed, however, that the relationship is not sufficiently close to justify their classification as rickettsiae and they may be considered as lying between them and the bacteria.

THE PLEUROPNEUMONIA-LIKE ORGANISMS[36]

The first of an apparently more or less homogeneous group of highly pleomorphic, filterable microorganisms to be described is the causative agent of *bovine pleuropneumonia*. Since its first study in 1898 a number of similar forms, both parasitic and saprophytic, have been found. For want of a better name they have been called the pleuropneumonia group or pleuropneumonia-like organisms, though none of the rest produces pleuropneumonia in cattle. Their relationship to other microorganisms is almost completely unknown and they have been grouped with the viruses by some because of their filterability, with the rickettsiae by others because of a predilection for an intracellular existence in the host, and, as indicated earlier, by still others they are grouped with the actinomycetes.

Morphology and Staining. There are probably few if any other microorganisms which are as highly pleomorphic as these forms. Impression preparations of colonies show granules of various sizes, filaments which may be branched and contain streaming protoplasm, balloon and disc-like structures, ring, club and star forms, and ameboid structures. Filaments are more numerous in recently isolated cultures and tend not to be formed in older stock cultures. The microscopic morphology of these forms is illustrated in a Giemsa-stained

[36] These microorganisms are discussed in detail in the review by Sabin: Bact. Rev., 1941, 5:1.

smear in Fig. 119 and the ring forms are well shown in the electron micrographs in Fig. 120. The marked pleomorphism is due in part to the fragility of the organisms, many of which are torn apart in making smears, and, as in the case of *Bacteroides funduliformis,* in part a consequence of modes of reproduction other than binary fission, *e.g.,* that of the development of round bodies which become nodular with outgrowths that segment into daughter cells. This process has been studied in detail by means of electron micrographs by Smith, Hillier and Mudd[37] and is illustrated in Fig. 122. Viable structures vary greatly in size and include ultramicroscopic elements which are filterable. Edward[38] describes experiments on filtration through gradocol membranes (p. 844) in which the concentration of organisms in an emulsion was reduced from 10^8 to 10^5 by passage through a membrane of APD 0.8 μ. This titer decreased
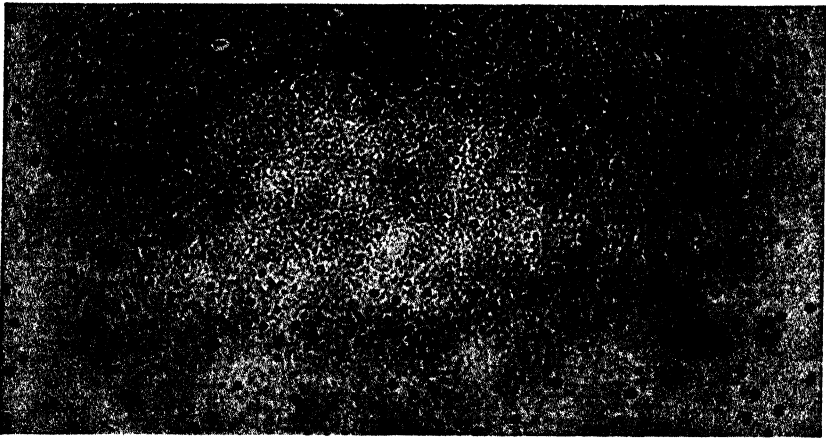
Fig. 119. Two-day culture of Type A pleuropneumonia-like organism from mice in serum-dextrose broth. Note the rings and elementary bodies. Giemsa; $\times$ 1000 (Sabin, Bact. Rev.).

progressively with membranes of decreasing pore size but complete retention did not occur until a membrane of 0.33 μ was used. In contrast to this, the end point is usually sharp in the filtration of viruses. On the basis of these observations the diameter of the smallest viable elements was estimated to be 165 to 247 mμ. Similar estimates vary somewhat for other strains; the agent of pleuropneumonia, for example, is 125 to 175 mμ.

These forms cannot be found in tissues by any method of staining. They are not demonstrable in smears stained by the usual aniline dyes, but are stained by certain polychrome stains, Giemsa and Castaneda's rickettsia stain. Very thick films prepared from the sediment of centrifuged cultures are gram-negative.

On primary isolation or change to a slightly different culture medium, broth cultures sometimes, but not always, show no detectable evidence of growth and may be carried along by "blind passage" for several transfers before it appears. Some strains show a uniform opalescence, others a granular type of

[37] Smith, Hillier and Mudd: Jour. Bact., 1948, 56:589.
[38] Edward: Jour. Path. Bact., 1940, 50:409.

growth. The characteristic of some strains to grow as small colonies, appearing as flakes attached to the side of the tube, is of some differential value. In any case, visible evidence of growth is very slight and almost all workers with these organisms have found it necessary to carry along an uninoculated tube of medium for comparative purposes.

The colonies upon an agar surface are usually not detectable until after two or three days' incubation. They are most readily observed in stained agar preparations. A square of agar is cut from a suspicious area on the plate and placed on a slide. It is covered with a coverslip on which an alcoholic solution of methylene blue and azure has been dried, and the space between the cover-

Fig. 120. Electron micrographs of the ring forms of the pleuropneumonia-like organisms (Lilly Research Laboratories).

slip and the slide filled with melted paraffin.[39] The average colony size of different strains ranges from 0.01 to 0.6 mm., rarely exceeding the larger figure. The colonies usually have a dark center with a lighter margin, and may appear coarsely or finely granular. Upon higher magnification a foam-like structure may be observed which is composed of the balloon-like forms. Oily droplets may be found in the colonies of some strains; these have been found, in some cases at least, to be largely cholesterol already present in the medium.[40] Possibly the pseudo colonies which have been observed[41] on uninoculated serum agar are of similar nature; it is said that these are readily differentiated from true colonies by experienced observers.

Physiology. These microorganisms require infusion or digest media enriched by the addition of serum or ascitic fluid in relatively large amounts.

[39] Dienes, Ropes, Smith, Madoff and Bauer: New England Jour. Med., 1948, 238:509.
[40] Partridge and Klieneberger: Jour. Path. Bact., 1941, 52:219.
[41] Brown, Swift and Watson: Jour. Bact., 1940, 40:857.

Various workers add from 10 to as high as 40 per cent serum; 50 per cent and over is inhibitory for some strains. Some have included boiled blood and fresh serum, and small amounts of glucose facilitate primary isolation of some strains. In some cases these organisms tolerate a relatively wide pH range, but others die out at pH 7.0 and below and require a pH of 7.8 to 8.0 for growth. The saprophytic varieties will grow at 22° C. with an optimum at 30° C. but the parasitic ones require 37° C. Growth occurs both aerobically and anaerobically, but is less abundant with most strains under anaerobic conditions. Fairly heavy inocula are required, 0.1 to 0.2 ml. of minced tissue in primary isolation, the transfer of similar amounts of broth cultures, and in transfer from agar cultures a small section of the medium is cut out and dropped into liquid media or streaked on another plate. These organisms may also be cultured on the chorioallantoic membrane of the developing hen's egg.[42] Some sugars, notably
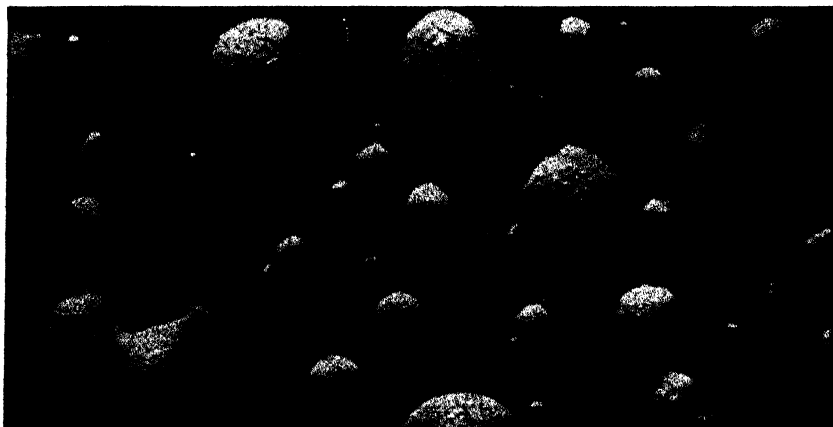
Fig. 121. Colonies of a Type B pleuropneumonia-like organism from mice. Three days' incubation; × 100. (Sabin, Bact. Rev.)

glucose, are fermented but the pH seldom drops below 7.0 owing to the death of the organisms at this point; the addition of other sugars may inhibit growth. In general, fermentations and other biochemical characteristics have no differential value.[43] The heat resistance of some strains is of the same order as that of most bacteria, while others appear to be more frail and are killed by exposure to 45° C. for fifteen minutes. Cultures are best preserved at incubator temperature in media containing no added sugar; when sealed with petrolatum the organisms may remain viable for a month or more.

Varieties, Strains or Species. Strains of these organisms have been isolated from a variety of sources. Those which are parasitic are definitely set off from the saprophytic types, and differentiation or identification within these groups has been made in part on source and pathogenicity, and in part on an immunological (agglutination) basis.

Bovine Pleuropneumonia. As indicated above, the causative organism of pleuropneumonia of cattle was the first of these forms to be isolated and

[42] Swift: Jour. Exp. Med., 1941, 74:557.
[43] *Cf.* Warren: Jour. Bact., 1942, 43:211.

studied. The disease is found all over the world except in India, Western Europe and North America; it has been imported into this country a number of times but was finally eradicated by slaughter of infected animals and has not occurred since 1892.

The natural disease in cattle is characterized by extensive consolidation and subpleural effusion in either or both lungs and the microorganism is present in large numbers in the serous exudate. It spreads slowly in herds and may take an acute form with death within a week or a chronic form with walling off of the foci of infection. There is occasional joint involvement in young animals.
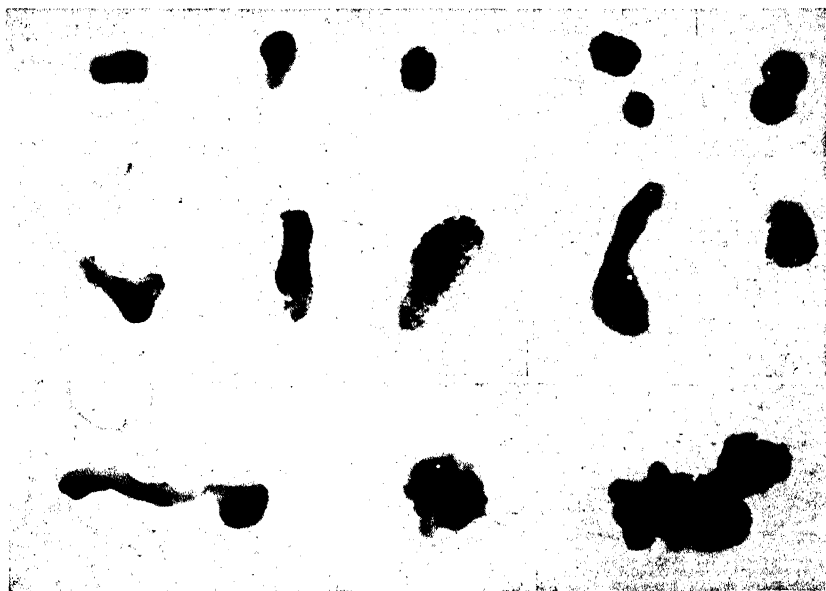
Fig. 122. The reproductive process of pleuropneumonia-like organisms. Both small round cells and rod-shaped cells, some of which have rounded enlargements, are shown. The round bodies develop from the rod-shaped cells in this way as illustrated in the lower left. The round bodies enlarge and the surface becomes nodular (lower center), and out growths occur and segment into multiple daughter cells (lower right). The round bodies are not spores and do not have the characteristic spore cell wall. Preparations fixed in osmic acid; × 15,000. (Rearranged from Smith, Hillier and Mudd, *loc. cit.*, by Dr. Smith.)

The natural disease has not been reproduced in cattle by either inoculation of infectious serous exudate or of cultures, but an extensive edema develops and spreads from the site of inoculation, and there is a febrile reaction and sometimes death.

The organism is apparently completely non-pathogenic for the usual experimental animals and for man; it has been reported that cultures in sheep- or horse-serum media are infectious for sheep and goats, while those in bovine-serum media are not.

The name *Asterococcus mycoides* was given the organism by Borrel and Dujardin-Beaumetz but has not been generally used. Sabin[36] has proposed the name *Bovimyces pleuropneumoniae*. In any case, strains obtained in different localities and at different times appear to be very similar if not identical.

Contagious Agalactia of Sheep and Goats. Despite its name this disease is a generalized infection which affects both males and females. It occurs only in parts of southern Europe and North Africa; agalactia or mastitis of these animals in this country is of other etiology.

The lesions occur in the joints and eyes and in the mammary glands of females. The microorganism is present in the blood early in the disease and later may be isolated from the affected regions and from the mammary secretions. The second member of the group to be studied, it was isolated by Bridré and Donatien in 1923. It is morphologically and biochemically very similar to the pleuropneumonia organism and other members of the group, but is immunologically distinct and of characteristic pathogenicity. Sabin[36] has suggested that it be called *Capromyces agalactiae.* The disease is readily reproduced by inoculation of sheep and goats with cultures.

Canine Variety. Organisms of the pleuropneumonia group have also been found in dogs. Shoetensack reported in 1934 the cultivation of an organism of this type from the purulent nasal discharge of dogs ill with distemper which he called *Asterococcus canis.* In later studies other organisms were found and there appeared to be two types, immunologically distinct from one another, which he called Type I and Type II respectively. Sabin[36] proposed that these be called *Canomyces pulmonis I* and *Canomyces pulmonis II.* Their postulated etiological relationship to distemper, generally regarded as a virus disease, is not established.

Varieties from Rats. A series of pleuropneumonia-like organisms has been isolated by Klieneberger and her associates[44] from the respiratory tract and elsewhere in normal and diseased rats. Others have isolated similar organisms from rats exhibiting polyarthritis and swollen extremities. These comprise, with a single exception, the "L" series of strains in which the strains are designated by subscript numerals. There now appear to be three distinct types, differentiated on a biological and immunological basis, viz., L_1, L_3 and L_4. The strain L_2 was isolated from a guinea pig and insufficiently studied before loss of the culture at the outbreak of war. The strain described as L_5 is immunologically identical with mouse type A discussed below; L_6 has been insufficiently studied; and L_7 has been found to be identical with L_4.

Considerable interest has attached to the apparent association of L_1 with *Actinomyces muris-ratti*,[45] Klieneberger has been able to isolate L_1 from many strains and stock cultures of *A. muris-ratti* and it will be recalled that this fungus is a natural parasite of the nasopharynx of rats and mice. It is maintained by Klieneberger[46] that L_1 and the fungus coexist as symbionts since she has been able to separate the two on the basis of differential resistance to heat and aging, has observed L_1 in the rat in the absence of *A. muris-ratti*, and has carried L_1 strains through many transplants without reappearance of the

[44] Klieneberger: Jour. Path. Bact., 1935, *40*:93; *ibid.*, 1936, *42*:587; Klieneberger and Steabben: Jour. Hyg., 1937, 37:143; Klieneberger: Jour. Hyg., 1940, *40*:204.

[45] It may be noted that the name *Streptobacillus moniliformis* continues to be used exclusively in the pleuropneumonia group literature in spite of its impropriety and almost always with no mention of its synonyms. The unnecessary confusion introduced is unfortunate. See page 670.

[46] In several papers; *Cf.* Klieneberger: Jour. Hyg., 1942, 42:485.

actinomycete. Dienes,[47] however, has taken the view that L_1 and *A. muris-ratti* represent but stages in a cyclogeny.

These L forms are associated, causally in some instances at least, with relatively mild, chronic affections of rats in which a not uncommon manifestation is joint involvement and polyarthritis. They closely resemble the other organisms of the group morphologically and culturally, forming a subgroup on the basis of pathogenicity, and differentiated from one another immunologically. Sabin[36] has grouped L_1, L_3, and L_4 under a single genus and suggested the names *Murimyces streptobacilli-moniliformis*, *Murimyces pulmonis* and *Murimyces arthritidis* respectively for them.

Varieties from Mice. Sabin[36] divides the pleuropneumonia-like organisms found in mice into five types, designated as A, B, C, D and E. Type A is found in normal mice, once in the brain and frequently in the eyes, nasal mucosa and lungs of carriers. On intracerebral inoculation of mice an ataxia is produced which is characterized by a turning or rolling of the body. The brain lesions and symptoms arising from them are attributable to the action of a soluble toxin produced by the microorganisms.[48] An immunologically identical organism was isolated by Findlay *et al.* from mice affected with "rolling disease" and designated L_5. Type B has likewise been found in normal mice and is not only immunologically distinct but produces a progressive arthritis almost exclusively when parenterally inoculated into mice. Types C, D and E are similar in their pathogenicity but are immunologically distinct; they have not been studied as extensively as some of the others. These murine forms are, like the L forms, grouped in a single genus by Sabin,[36] type A being given the name *Musculomyces neurolyticus*, type B the name *Musculomyces arthrotropicus*, and types C, D and E grouped in the single species *Musculomyces histotropicus*.

Pleuropneumonia-like Organisms of Man.[49] Though there is considerable evidence suggesting that certain streptococci are associated with some types of arthritis in man (p. 377), the etiology of human arthritis is far from clear. The pleuropneumonia-like organisms have assumed some interest in this connection, largely because of the frequency of occurrence of progressive polyarthritis in both naturally and artificially infected rats and mice. The association of L_1 strains with *A. muris-ratti* is of possible interest in view of the arthritic manifestations of epidemic disease of the Haverhill fever type and of sporadic ratbite fever of fungous etiology (p. 670).

Microorganisms of this group have been found by a number of workers in the genital tract. They appear to occur with some frequency in the normal vagina and cervix, in 58 of 222 routine specimens in the series reported by Dienes *et el.*,[49] but they have also been found associated with inflammatory conditions of the cervix. In the male, however, they have been found largely in association with pathology of the genito-urinary tract. Dienes *et al.* reported that all of 58 patients from whom cultures were recovered had urethritis,

[47] Dienes: Jour. Inf. Dis., 1939, 65:24. Also Heilman: *ibid.*, 1941, 69:32, 45.

[48] Sabin: Science, 1938, 88:189, 575.

[49] See the review and discussion by Dienes, Ropes, Smith, Madoff and Bauer: New England Jour. Med., 1948, 238:509, 567.

prostatitis or cystitis. In relation to the possible association of these micro-organisms with arthritis in man, it is of interest that 18 of this series had an acute type of arthritis, 9 had simultaneous urethritis, conjunctivitis and arthritis, the syndrome characteristic of Reiter's disease, and in two instances pleuropneumonia-like organisms were recovered from synovial fluid. Observations such as these suggest that they may be pathogenic for man.

Saprophytic Varieties. Pleuropneumonia-like organisms presumably living a saprophytic existence in nature have been found by Laidlaw and Elford[50] in raw London sewage. They closely resembled the parasitic forms both morphologically and culturally, and fell into three immunological groups which were designated types A, B and C. All were non-pathogenic for experimental animals. Similar forms have been found in Germany[51] in compost and other types of decomposing organic matter. Sabin[36] has proposed that these saprophytic forms be named *Sapromyces laidlawi.*

DONOVANIA GRANULOMATIS

The disease granuloma inguinale (granuloma venereum) is not to be confused with lymphogranuloma inguinale of virus etiology (p. 882). It is characterized by a slowly progressive ulceration in the genital region and rarely elsewhere. The initial lesion is a swelling, often in the groin as a bubo, which ruptures. Daughter lesions appear which are at first discrete and then spread slowly and coalesce, and the process may eventually involve the skin of the groin, genitals, buttocks and lower abdomen and the patient develops a strong fetid odor. Little effective immunity appears to be developed, at least not sufficient to appreciably arrest the progress of the infection.

Bacillary bodies, stained by Wright's stain, were observed by Donovan in 1905 in smears from lesions or in biopsy material, and have long been known as Donovan bodies. The Donovan body has been cultivated in the yolk sac, but not on the chorioallantois, of the developing chick embryo by Anderson, De Monbreun and Goodpasture[52] and in enriched media such as beef heart infusion by Dunham and Rake,[53] and is thus shown to be a cultivable microorganism to which the name *Donovania granulomatis* has been given.

The morphology of this microorganism in culture has been studied by Rake and Oskay,[54] and it is described by these workers as a short, plump bacillus 1.5 μ to 4.5 μ in length and 0.8 μ to 1.4 μ in breadth, gram-negative and showing prominent polar granules. Prior to cell division the elongated bacillary forms tend to become curved, and may remain attached after division to give rise to chains of bacilli, the coiled filaments often seen in the usual stained preparations. The relatively heavy encapsulation observed in preparations from lesion material and the mucoid character of yolk sac cultures and initial cultures on artificial media diminish with continued culture. Aside from its highly fastidious growth requirements, *D. granulomatis* closely resembles Friedländer's

[50] Laidlaw and Elford: Proc. Roy. Soc. (London) Ser. B, 1936, *120*:292.
[51] Seiffert: Centralbl. f. Bakt., I Abt. Orig., 1937, *139*:337; *ibid.*, 1937, *140*:168.
[52] Anderson, De Monbreun and Goodpasture: Jour. Exp. Med., 1945, *81*:25.
[53] Dunham and Rake: Amer. Jour. Syph. Gonorrhea Ven. Dis., 1948, 32:145.
[54] Rake and Oskay: Jour. Bact., 1948, *55*:667.

bacillus, and has been found by Rake[55] to be closely related immunologically to this bacterium and to *Bact. coli* and *Bact. aerogenes.*

Prior to the isolation in pure culture of *D. granulomatis,* its causal relation to granuloma inguinale was only suggested by association. It was shown by Anderson, Goodpasture and De Monbreun[56] that material from yolk sac cultures gave a skin reaction in persons having the disease, and a mucoid substance from infected yolk sac, possibly a polysaccharide, gave precipitin and complement-fixation reactions with sera from patients. The specificity of these reactions is, however, open to question in that apparently specific complement fixation was given by persons with chronic, non-specific ulceration (*viz.,* varicose) studied by Rake and his co-workers and attributed by them to the serological relation of *D. granulomatis* to coliform bacilli. Later Greenblatt *et al.*[57] were able to produce the disease in two human volunteers, one inoculated by a subcutaneous transplant of biopsy material, and the other by the subcutaneous inoculation of yolk sac culture. Though it has not yet been possible to infect experimental animals with *D. granulomatis,* it seems established that this microorganism is the etiologic agent of granuloma inguinale.

The general tendency to regard this disease as venereal is based in large part on the location of the lesions. There is little or no direct evidence that it is transmitted primarily by sexual contact and, in fact, its occurrence in both marital partners is uncommon. The probable incubation period of one to four weeks is not excessively long and should not greatly obscure histories of contact. It has been suggested that there is great individual variation in susceptibility and that natural resistance is usually of a high order. The disease is associated with uncleanliness and in this country occurs for the most part in the Negro of low economic status in the southeastern States, but it occurs elsewhere also. It is estimated that there are 5000 to 10,000 cases in the United States and it has been found to constitute 2 to 3 per cent of venereal disease in Negro recruits. In general, however, the epidemiology of granuloma inguinale is as yet very poorly understood.[58]

[55] Rake: Jour. Bact., 1948, 55:865.
[56] Anderson, Goodpasture and De Monbreun: Jour. Exp. Med., 1945, 81:41.
[57] Greenblatt, Dienst, Kupperman and Reinstein: Jour. Ven. Dis. Inf., 1947, 28:183.
[58] See the discussion by Clarke: Jour. Ven. Dis. Inf., 1947, 28:189.

BACILLUS—THE SPORE-FORMING AEROBES

The spore-forming rod-shaped bacteria are divided into two groups on the basis of their relation to atmospheric oxygen. The Bacilli are the aerobic forms and the anaerobic types are designated Clostridium (Chap. 28). A very large number of species of Bacilli have been described, the majority of them from soil and dust. Two species, *Bacillus alvei* and *Bacillus paraalvei,* cause foulbrood, a disease of bees. *Bacillus subtilis* infects human beings only rarely, and, with this exception, *Bacillus anthracis* is the only member of this large group that is pathogenic for man.

BACILLUS ANTHRACIS

Primarily a disease of lower animals transmissible to man, anthrax (splenic fever; Fr. *charbon;* Ger. *Milzbrand*) is of particular historical interest, for it was in his study of this disease that Koch provided the first demonstration of the causal relation between a specific bacterium and an infectious disease. The bacillus had been observed in the blood and organs of animals dying of anthrax by Davaine and Rayer in 1850 and by Pollender in 1855. In 1887 Brauell transmitted the disease by the inoculation of blood from infected animals. Conclusive demonstration of the causal relation between the bacilli and the disease, however, was the work of Koch, who in 1877 cultured the bacillus on the aqueous humor of the ox's eye, described its life history, and reproduced the disease with a pure culture of the microorganism. The importance of this discovery to the development of bacteriology has been discussed elsewhere (Chap. 1).

Morphology and Staining. The anthrax bacillus is one of the largest of the pathogenic bacteria and ranges from 4.5 to 10 μ in length and from 1 to 1.25 μ in breadth. The ends of the rods are often concave and somewhat swollen so that the appearance of a chain of anthrax bacilli has often been compared to a jointed bamboo fishing rod. The cells occur singly and as end-to-end pairs or short chains in the body, but in culture long chains are formed. Unlike most of the sporulating aerobic bacilli they are non-motile.

Capsules may be found on the bacilli in smears from an infected animal but are not found in culture except on media rich in animal protein, such as serum agar. The capsular material is not polysaccharide as it is in most bacteria, but is a high molecular weight polypeptide composed exclusively of d(-)glutamic acid (the "abnormal" stereoisomer).[1] This is a point of particular interest, for it is the first demonstrated natural occurrence of d(-)glutamic acid and of a polypeptide composed of a single amino acid. There is, in addition, a poly-

---

[1] *Cf.* Hanby and Ryden: Biochem. Jour., 1946, 42:297.

saccharide haptene present in the cell substance of the bacilli, which may be isolated from these bacilli; it appears to be the same in both virulent and avirulent strains and its immunological function is not clear.[2]

The anthrax bacillus also differs from most other aerobic pathogenic bacteria in that it forms spores which are visible as refractile bodies either free or located centrally within the cell. Their diameter does not exceed that of the vegetative cell and hence the spore-containing rod is not distorted. Spores are formed most abundantly at 32° to 35° C. and only under aerobic conditions, *i.e.*, not in the circulating blood of infected animals. Germination of the spore is usually polar, that is, parallel with the long axis, but may be rarely equatorial.

The bacilli stain readily, but often unevenly, with the usual aniline dyes. The granular material within the cell consists of fat, volutin or glycogen.
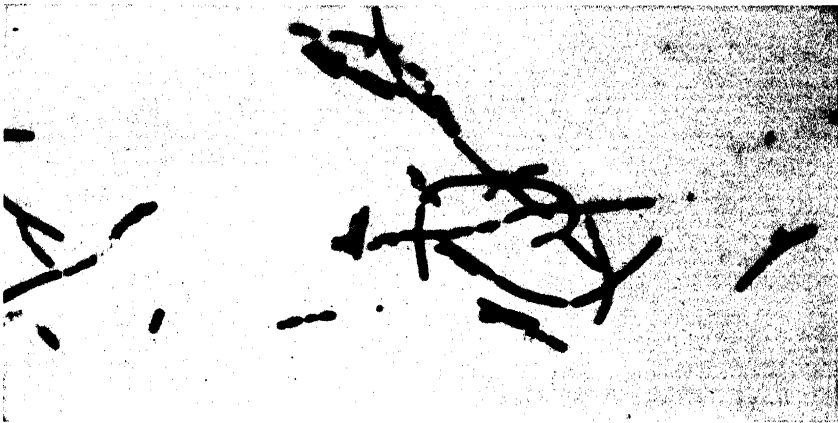
Fig. 123. *Bacillus anthracis*, forty-eight-hour culture on nutrient agar. Crystal violet stain. The spores appear as unstained areas. Note the typical arrangement of the bacilli in coiled chains. × 1200.

They are gram-positive. Churchman[3] has observed a reversal of the Gram reaction when aqueous gentian violet, acriflavin or acriviolet is added to suspensions of young cultures of *Bacillus anthracis*; a considerable proportion of the bacilli then become gram-negative. It has been concluded that the bacillus is made up of a gram-positive outer layer or cortex and a gram-negative medulla. When the former is destroyed the latter comes into view and the gram-negative bacilli are considerably smaller than the gram-positive cells. The spores are stained with difficulty, and after staining with hot carbol fuchsin are equally difficult to decolorize; hence the vegetative cells may be decolorized and stained with a contrasting dye.

The colonies of the anthrax bacillus are irregular and have a curled or hair-like structure, giving what is sometimes called a "Medusa head" appearance. On microscopic examination tangled coils of long chains of bacilli may be found. This colonial appearance is closely simulated by *Bacillus subtilis* and some other related saprophytic, aerobic, spore-forming bacilli.

[2] Ivanovics: Ztschr. f. Immunitätsf., 1940, 97:443.
[3] Churchman: Jour. Exp. Med., 1927, 46:1007.

Physiology. The anthrax bacillus grows readily upon all the ordinary laboratory media, and growth is not improved by the addition of enriching substances. It can be grown on simple synthetic media; thiamine, magnesium, iron and calcium are required together with a source of energy, and uracil, adenine, guanine and manganese markedly stimulate growth.[4] Growth occurs at temperatures as high as 41° to 43° C., with an optimum at 37° C. These bacillus are aerobic and facultatively anaerobic. Dextrose and trehalose are fermented rapidly but without gas production. Sucrose, maltose and some other carbohydrates are fermented less rapidly; lactose, galatose, mannitol, dulcitol, rhamnose and xylose not at all. Gelatin is slowly liquefied but indol is not formed, nitrate is not reduced, and little or no hydrogen sulfide is produced. Milk
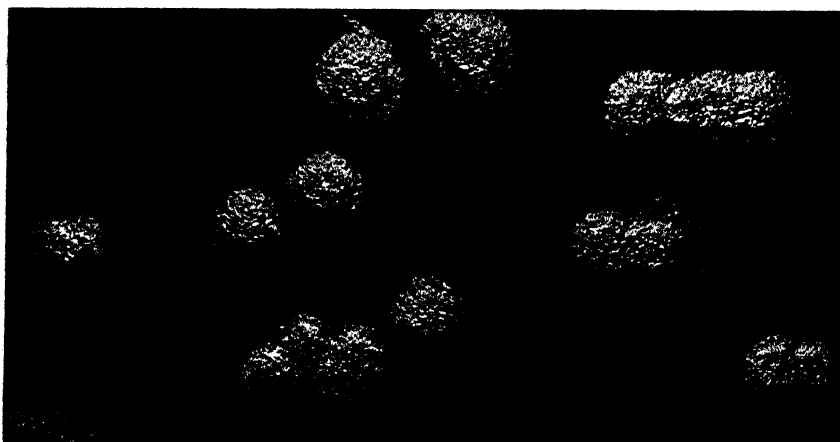
Fig. 124. Colonies of *Bacillus anthracis* on nutrient agar. Twenty-four-hour culture. Note the large size and coarse texture suggestive of R variants. × 3.

is feebly acidified and is curdled by a rennet-like ferment, and the casein slowly peptonized. On potato a gray, furry growth is produced; spores are formed in particular abundance on this medium.

No cultural or biochemical characteristics serve to differentiate the anthrax bacillus from the very similar nonpathogenic saprophytic sporulating bacilli; animal inoculation to determine pathogenicity is essential.[5]

The vegetative cells of the anthrax bacillus display the usual degree of resistance to deleterious influences, but the spores are relatively highly resistant, although not so resistant as the spores of *Bacillus subtilis* and related forms. Graham-Smith[6] has found that spores exposed to daylight at room temperatures would germinate after twenty-two years but not after twenty-two and one-half years; spores dried on canvas and kept in envelopes would not germinate after thirty-five years. Anthrax spores are usually destroyed by boiling for ten minutes and by dry heat at 140° C. for three hours. Their resistance to disinfectants is variable, for 0.1 per cent mercuric chloride may fail to kill them in seventy hours, while those disinfectants which are oxidizing agents

[4] Brewer *et al.*: Arch. Biochem., 1946, *19*:77.
[5] See, for example, Stein: Amer. Jour. Vet. Res., 1944, 5:38.'
[6] Graham-Smith: Jour. Hyg., 1941, *41*:496.

are much more effective; 3 per cent hydrogen peroxide kills in one hour and 4 per cent potassium permanganate in fifteen minutes. In the animal carcass vegetative cells are destroyed during anaerobic putrefactive changes in seventy-two hours, but spores are viable under such circumstances for at least nine months.[7] In the soil anthrax spores may remain viable for many years.

Variation. The rough variant is the virulent and naturally occurring form of *B. anthracis*. It was early noted by Pasteur that prolonged cultivation of these bacilli at higher than optimum temperatures, 42.5° C., resulted in a loss of virulence and the appearance of asporogenous variants. A number of different types of variants are produced by such cultivation at high temperatures or in the presence of dilute antiseptics. Smooth and mucoid types of colonies have been observed, and some of these variants may be non-spore-forming. Virulence, however, is associated with the presence of a capsule, or the ability to form one, rather than the ability to form spores, for both asporogenous virulent strains and spore-forming avirulent strains may be found. These probably do not occur in nature, however.

Toxins. The anthrax bacillus forms neither soluble toxin nor endotoxin and its virulence appears to be associated with the glutamyl polypeptide capsule. In this connection it is of interest that the capsular substance is not hydrolzed to an appreciable extent by the proteolytic enzymes of the body and is, in fact, excreted quantitatively by the rabbit. However, Watson *et al.*[8] have found that an inflammatory factor, not identical with the capsular material, is produced which gives rise to tissue damage similar to that observed in infections. The activity also resembles hyaluronidase in some respects and interferes with the blood-clotting mechanism. Sterile extracts of anthrax lesions, presumably containing this activity, are capable of producing histopathological changes comparable to those of the disease.[9] A hyperglycemia occurs during infection and in its terminal stages the disease superficially resembles magnesium poisoning; a curious observation is that the injection of calcium salts appears to have some protective effect.

The cause of death in anthrax still remains obscure. In typical anthrax septicemia bacilli are found in immense numbers clogging the capillaries and it was early supposed that death resulted from tissue anoxia due to mechanical interference with the circulation. Death may occur, however, in the absence of great numbers of bacilli in the blood and this hypothesis is not tenable.[10]

Pathogenicity for Lower Animals. In nature anthrax is primarily a disease of cattle and sheep; horses and swine are susceptible, but are less commonly affected. Wild deer and other gregarious herbivora are liable to occasional outbreaks. The smaller rodents are very sensitive to inoculation. Rabbits, guinea pigs and white mice are susceptible in that order, and are fatally affected by the subcutaneous introduction of a very small number of virulent bacilli. The white mouse may succumb to inoculation with a single bacillus of a highly virulent strain. Carnivorous animals, though possessing

---

[7] Stein: Vet. Med., 1947, 42:13.

[8] Watson *et al.*: Jour. Inf. Dis., 1947, 80:121.

[9] Cromartie, Watson, Bloom and Heckly: Jour. Inf. Dis., 1947, 80:14.

[10] See the discussion by Bloom, McGhee, Cromartie and Watson: Jour. Inf. Dis., 1947, 80:137.

greater resistance than the herbivora, are nevertheless susceptible, as several epidemics in zoological gardens involving leopards, lions, pumas, bears and other animals have shown. Certain animals possess a marked natural resistance to anthrax. Rats are quite resistant, especially the white rat, only about 14 per cent of the latter dying as the result of inoculation. The mature dog is only slightly susceptible. Birds, especially pigeons, can be infected, but not easily. Frogs are completely resistant but toads are very susceptible.

The route by which the bacilli enter the body exerts an important influence in both experimental and natural infections. Subcutaneous inoculation is the method most commonly practiced in experimental work, and is almost uniformly fatal with the ordinary small laboratory animals. Feeding experiments show that administration of spore-free cultures even to highly susceptible animals is without result, owing to the destruction of the bacilli in the stomach. The feeding of spores, on the other hand, leads to infection of the more susceptible species, although not so certainly as subcutaneous inoculation; resistant species, such as swine, may be infected through the alimentary tract only with difficulty. Infection through the respiratory tract is possible in the experimental animal but is probably almost unknown in the lower animals under natural conditions.

In highly susceptible animals the disease is acute and runs a rapid course; the case fatality in cattle and sheep is about 80 per cent. It presents all the characteristics of a typical septicemia and local manifestations may be almost entirely absent. Enormous multiplication of the bacteria takes place in the blood and internal organs, and sections through the liver or spleen show the capillaries gorged with masses of bacteria. The spleen is of a deep-red color and greatly enlarged, hence the name splenic fever. The more resistant animal species do not develop this generalized infection, but the bacteria remain localized in an abscess or carbuncle and fail to spread through the body. This is the case with the dog and in some forms of infection in man. In this respect anthrax furnishes an illustration of the general rule that when a bacterial invasion meets slight resistance from the animal tissues an abundant multiplication of the bacteria occurs throughout the body, while the possession of high powers of resistance is accompanied by a pronounced local reaction. Man stands perhaps midway in susceptibility between the dog and the sheep.

Under natural conditions cattle and sheep are infected through the alimentary tract by swallowing spores while grazing in infected pastures. As has been pointed out, spores are able to retain their vitality in soil for a long period, and pastures once infected may infect cattle after a lapse of as many as thirty years. Hides imported from China and other countries where the disease prevails are not uncommonly contaminated with anthrax spores; in the United States several outbreaks of anthrax among cattle with some consequent cases of human infection have been traced to the overflowing of pasture land by streams receiving the drainage of tanneries.

Cattle may also occasionally be infected by direct contact through wounds, abrasions and other injuries to the skin; but alimentary infection is by far the most common. Anthrax has been experimentally transmitted to susceptible animals by biting flies of various species that had previously fed on animals

dying from anthrax.[11] The bacilli persist in the insects for only a short time.

Pathogenicity for Man. Three routes of infection of human beings are known: (*a*) through the skin, (*b*) through the respiratory tract and (*c*) through the alimentary tract. The bacillus is almost always transmitted to man through the agency of the lower animals rather than through other human beings. The persons most commonly affected are those having to do with cattle and their products, such as butchers, shepherds and herdsmen, handlers of hides, hair and fleeces. In the United States there were 357 cases of anthrax and 52 deaths in 1934–38, and in 1939–43 408 cases and 33 deaths.[12] The incidence of cases of infection from wool and hair increased nearly five-fold in the second five year period and over 90 per cent of the infections contracted in tanneries were from goat skins. During the first World War less efficient preliminary disinfection of hides and bristles permitted the introduction of anthrax-contaminated articles from parts of Asia and South America, and a striking increase in anthrax occurred from the use of shaving brushes—the bacilli were isolated from brushes purchased in the open market in some instances. The bacilli are destroyed on such brushes by soaking in 10 per cent formalin at 110° F. for four hours. Laboratory infections, sometimes fatal, have been known to occur with pure cultures of the anthrax bacillus. The case fatality of anthrax in man is probably about 20 per cent.

Cutaneous Anthrax (Malignant Pustule). The most common form of anthrax in the human subject is due to skin infection, and usually takes the form of a localized boil or abscess, which often heals spontaneously but may progress into a septicemic condition unless checked by incision or other surgical procedure. Owing to the relatively high resistance of man septicemia does not often occur, especially if the carbuncle be incised and thoroughly drained. Lesions of all sizes may be produced, from a minute pustule to a large abscess.[13]

Pulmonary Anthrax. The pulmonary form of anthrax due to inhalation of the microorganisms is the most dangerous, although not the most common, variety of the disease in man. It is an occupational disease among those who handle and sort wools and fleeces and contract the infection by inhalation of spores set floating in the air from the infected material; pulmonary anthrax is known in England as "woolsorters' disease." It is characterized by many of the symptoms of pneumonia and often passes into a fatal septicemia. Experimental air-borne anthrax was studied intensively by Young, Zelle and Lincoln[14] and it was found that a very few spores sufficed in the case of virulent strains, entering the tissues from the alveoli via the lymphatic system. The inhaled spores produced only slight local reaction except for a clogging of the capillaries in the terminal stage of the disease.

Intestinal Anthrax. The alimentary tract, although the usual path of infecfection in cattle, is very rarely so in man. A few instances are on record of

[11] Mitzmain: Hyg. Lab. Bull. No. 94, 1914. See also Kraneveld and Djaenoedin: Nederland. Indische Blad. Diergeneesk., 1940, 52:339.

[12] Report, Committee on Industrial Anthrax: Amer. Jour. Pub. Health, 1945, 35:850.

[13] For a discussion of cutaneous anthrax see Hodgson: Lancet, 1941, ii:811; Gold: Arch. Int. Med., 1942, 70:785; Ellingston, Kadull, Bookwalter and Howe: Jour. Amer. Med. Assn., 1946, 131:1105.

[14] Young, Zelle and Lincoln: Jour. Inf. Dis., 1946, 79:233.

intestinal anthrax contracted through the medium of spore-infected food. Such cases occur among workers with animal products, and have probably been due to lack of caution in handling food with uncleansed hands. Insufficiently cooked meat from anthrax-infected animals may also be a source of intestinal anthrax.

Immunity. The cause of the high natural immunity to anthrax possessed by the dog, the fowl and certain other animals has been the object of much experimentation, but no clear-cut explanation has as yet been found. The body fluids of some species manifest bactericidal powers toward the anthrax bacillus, but there is no concurrence between the degree of immunity and the anthracidal power of the blood serum. The blood serum of the highly susceptible rabbit is strongly bactericidal outside the body, but anthrax bacilli injected into the circulation seem to multiply freely in the blood stream. Blood taken
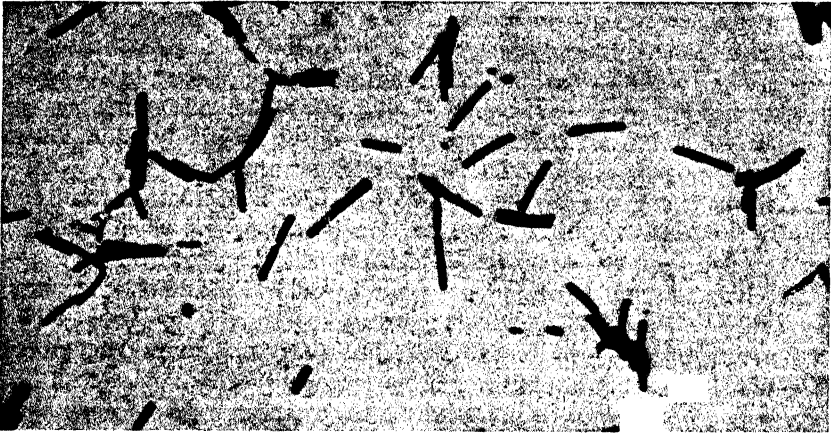
Fig. 125. *Bacillus subtilis*, twenty-four-hour culture on nutrient agar. Crystal violet stain. No spores have formed as yet. Note the typical arrangement of the bacilli. × 1200.

from the very resistant dog and fowl is practically devoid of bactericidal properties.

Acquired immunity to anthrax is a consequence of the presence of living, virulent bacilli in the body; suspensions of killed cells or completely avirulent bacilli do not produce an immunity. Pasteur devised a method for vaccinating cattle and sheep against anthrax which is dependent on the subcutaneous inoculation of attenuated cultures. Two vaccines were used. The "first vaccine" consisted of bacilli grown at 42.5° C. for fifteen to twenty days and would not always kill guinea pigs though still fatal for white mice. After twelve days a second inoculation was made with the "second vaccine" of bacilli cultivated at the higher temperature ten to twelve days, which would kill guinea pigs but not rabbits. Following inoculation with these two vaccines, a fully virulent culture could be injected with impunity. In spite of some accidents due to the use of imperfectly standardized vaccines, this method of protective inoculation has proved, on the whole, of great practical value. In France 30,-000 to 50,000 cattle and horses and 250,000 to 350,000 sheep are vaccinated

annually. Active immunization of rabbits and guinea pigs can also be effected by the injection of attenuated cultures, but with much greater difficulty.

The simultaneous inoculation of anti-anthrax serum and a spore vaccine (Sobernheim's method) has been quite extensively practiced in the United States in districts where anthrax is prevalent, and here, too, occasional infections with the vaccine occur. It has been found by Cromartie *et al.*[15] that the sterile extract from anthrax lesions which produces histopathological changes comparable to those found in the disease is an effective immunizing agent in experimental animals. The immunizing agent is, however, distinct from the inflammatory factor. It was early postulated by Ascoli that immunity to anthrax involves some process which retards capsule formation, and the evidence of
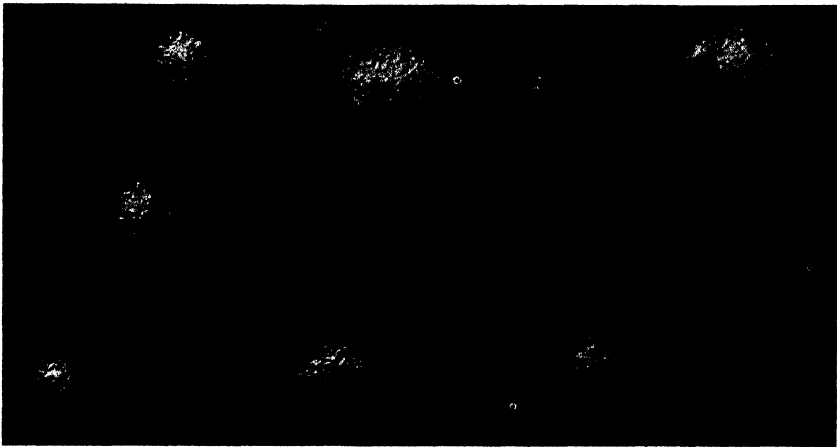
Fig. 126. Colonies of *Bacillus subtilis* on nutrient agar. Twenty-four-hour culture. Note the resemblance to colonies of the anthrax bacillus. × 3.

recent years is in accord with this hypothesis, but very little is known of the mechanisms involved.

The serum of actively immunized animals contains specific protective substances, and inoculation with such antisera confers some degree of passive immunity. Antisera have been used in the treatment of human anthrax with encouraging results. At the present time it is customary to treat anthrax cases with a combination of antiserum and arsenicals,[16] and recent evidence suggests that penicillin is of value.

Bacteriological Diagnosis of Anthrax. If the specimen is fresh and not grossly contaminated the anthrax bacillus may be found in gram-stained smears as a rule and is readily cultured on the usual nutrient media. Such a specimen may also be used for guinea pig inoculation, but it must be borne in mind that the sporulating obligate anaerobes will kill as rapidly as the anthrax bacillus. In any case the isolated culture must be tested for pathogenicity by

[15] Cromartie, Watson, Bloom and Heckly: Jour. Inf. Dis., 1947, 80:14.
[16] Cf. Amer. Pub. Health Assn. Yearbook, Suppl. Amer. Jour. Pub. Health, 1935, 25:No. 2; see also Cruickshank: Lancet, 1939, ii:681.

animal inoculation. The animal will die in thirty-six to forty-eight hours after subcutaneous inoculation of a very small amount of culture. A gelatinous infiltration will be found at the site of inoculation and the tissues of the animal contain enormous numbers of the bacilli; smear of cut spleen, for example, will show many of the large gram-positive bacilli. This demonstration of pathogenicity is sufficient for identification since none of the aerobic sporulating rods that resemble the anthrax bacillus is pathogenic for the guinea pig and similar experimental animals.

A precipitation test, the Ascoli test or thermo-precipitation test, is sometimes used to detect anthrax contamination of hides or other tissues. The specimen is

Fig. 127. Colonies of *Bacillus mycoides* on nutrient agar. Twenty-four-hour culture. × 3.

extracted with boiling water and the extract used as an antigen in a precipitin ring test with very high titer anthrax antiserum.

RELATED BACILLI

As indicated earlier, there are many species of aerobic sporulating bacilli closely related to and indistinguishable from the anthrax bacillus on any basis other than pathogenicity. The close morphological similarity led many of the early workers to describe "avirulent anthrax bacilli," "pseudo-anthrax bacilli," and "Bacillus anthracoides." There is, however, little basis for such differentiation. On the contrary, the vast majority of these bacilli are saprophytic soil forms and are commonly found as contaminants on plates because of the wide distribution of their spores in dust.

Of the commonly encountered forms, *B. subtilis, B. megatherium* and *B. cereus* are among the most familiar. *B. mycoides*, sometimes called *B. ramosus*, is closely related to *B. cereus* and is classified by Bergey (1948) as a variety of it rather than a separate species. There are some 33 species of Bacillus in all. The following portion of a key to the genus is abridged from Bergey (1948) and indicates the manner in which species are separated and defined. The close relation of the anthrax bacillus to the saprophytic forms is noteworthy.

Mesophilic, aerobic bacilli with spores ellipsoidal to cylindrical and central to terminal:
(I) Diameter of vegetative cells less than 0.9 μ (small cell variety)
    (1) Grow at pH 6.0; acetylmethylcarbinol formed
        (a) Gelatin hydrolyzed
            (i) Starch hydrolyzed; nitrate reduced to nitrite
                Bacillus subtilis
            (ii) Starch not hydrolyzed; nitrate not reduced to nitrite
                Bacillus pumilus
        (b) Gelatin not hydrolyzed
            Bacillus coagulans
    (2) No growth at pH 6.0; acetylmethylcarbinol not formed
        (a) Casein digested; urease not formed
            Bacillus firmus
        (b) Casein not digested; urease formed
            Bacillus lentus
(II) Diameter of vegetative cells 0.9 μ or more (large cell variety).
    (1) Acetylmethylcarbinol not produced
        Bacillus megatherium
    (2) Acetylmethylcarbinol produced
        (a) Saprophytic, usually motile
            Bacillus cereus
            Bacillus cereus var. *mycoides*
        (b) Pathogenic, non-motile
            Bacillus anthracis

These forms can be distinguished from one another on the basis of details of spore formation, differential fermentations and the like, and constitute stable types. Immunological investigation has confirmed the homogeneity of these types also. The immunological specificity of the spores appears to be different from that of the cell substance, and Lamanna[17] has found that four main types of the small cell group can be distinguished though differentiation of the large cell group by this means is not so satisfactory. Sievers[18] has reported similar results in studies of the specificity of the cell substance.

The pathogenicity of these forms is very slight at best but *B. subtilis* is occasionally responsible for infection, particularly of the eye, and rarely may produce a septicemia in the immature animal. Other bacteria of this group are occasionally found to have feeble pathogenic powers. Heaslip[19] isolated an aerobic sporulating bacillus which he called *Bacillus tropicus* by the inoculation of mice with blood from persons suffering from a mild infection in Australia called "coastal fever." This bacillus has also been found there as a natural parasite of the rat and the bandicoot. It appears to be very similar to a bacillus described by Scott many years before as *Bacillus seroficus*. *Bacillus alvei*, the cause of foulbrood of bees, is not pathogenic for man.

[17] Lamanna: Jour. Inf. Dis., 1940, 67:193, 203.
[18] Sievers: Jour. Bact., 1942, 43:305.
[19] Heaslip: Med. Jour. Australia, 1941, 2:536.

CLOSTRIDIUM—THE SPORE-FORMING ANAEROBES[1]

The group of anaerobic sporulating bacilli includes a variety of forms. Some of these, the anaerobic nitrogen-fixing bacteria, the butyl alcohol and acetone-producing forms and others, have been discussed earlier (Chap. 4). Still others, however, are pathogenic for man and lower animals, and the more important of these forms—*Clostridium tetani, Clostridium septicum, Clostridium welchii, Clostridium novyi, Clostridium histolyticum, Clostridium chauvei, Clostridium botulinum* and the non-pathogenic but common species *Clostridium sporogenes*—will be considered here.

The status of these bacteria as parasites is open to some question. They occur in the soil, in particular abundance in manured soils, and are found in the intestinal tract of man and animals. *Cl. welchii*, for example, is uniformly present in human feces, and the tetanus bacillus is often found (in up to 40 per cent of specimens examined) in the feces of domestic animals. It has been assumed by some that these bacilli are parasitic and their presence in the soil is a consequence of contamination. Although their numbers are unquestionably greatly increased by manuring and other forms of contamination, some have been found in virgin soils. It is perhaps best to regard them as essentially saprophytic soil forms that are capable of maintaining themselves in the large intestine.

None of these bacteria possesses any marked ability to invade the body tissues by itself. *Cl. botulinum* is apparently incapable of setting up an infection, while others, such as the tetanus bacillus, produce local infections when aided by traumatic injury to the tissues and frequently by the presence of other bacteria. Still others, such as the bacilli associated with gas gangrene, show pronounced invasive properties when once established, but the initial invasion is made possible by other factors, usually trauma and the presence of other bacteria.

The pathogenicity of the anaerobic bacilli is, rather, attributable to their ability to form powerful exotoxins, a property which is curiously confined to these bacteria, the diphtheria bacillus and possibly the Shiga dysentery bacillus. In the case of botulism the toxin is preformed outside the animal body and, since it is unique in that it is resistant to the digestive enzymes, enters the body by way of the alimentary tract and absorption into the tissues. In the other cases a focus of infection is established and the toxin formed at that point is disseminated through the body. In some instances, such as gangrene,

[1] These and other anaerobic bacteria are considered at length by Weinberg, Nativelle and Prevot: *Les Microbes Anaerobies*. Masson et Cie., Paris. 1937. The serological relationships of these bacteria are reviewed by McCoy and McClung: Bact. Rev., 1938, 2:47.

an extensive local destruction of tissue occurs, but in general the diseases caused by these bacilli are essentially toxemias.

It will be clear from the foregoing considerations that infections with the sporulating anaerobes are not common under ordinary circumstances, for in most instances traumatic injury is a preliminary to infection. On the battlefield, however, such injuries are common, and tetanus and gangrene are not infrequent complications of war wounds. Such anaerobic wound infections were prominent in the first World War, perhaps as a consequence of battles fought over the heavily manured fields of France. The occurrence of gaseous gangrene during the World War II among troops in North Africa where the desert soil is relatively free of such forms suggests that clothing may be a more important source of contamination than had been supposed.

Cl. histolyticum is microaerophilic, *i.e.*, it will grow in the presence of small amounts of oxygen, but the remainder of the forms considered here

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE MORE IMPORTANT PATHOGENIC ANAEROBES

	Spores	Cap-sule	Motil-ity	Prote-olysis	Fermentations			
					Dex-trose	Lac-tose	Su-crose	Exo-toxin
Cl. tetani	spherical, terminal	—	+	—	—	—	—	+++‡
Cl. septicum		—	+	+sl*	+	+	—	++
Cl. welchii		+	—	+sl	+	+	+	++
Cl. novyi	oval, sub-terminal	—	+	+sl	+	—	—	+++
Cl. histolyticum		—	+	+	a†	—	—	+
Cl. sporogenes		—	+	+	+	—	—	—
Cl. chauvei		—	+	+sl	+	+	+	++
Cl. botulinum		—	+	±	+	±	—	+++

* sl: relatively slight; † a: acid; ‡ strong, moderate and weak.

are obligate anaerobes. They may be isolated in pure culture by picking colonies from a shake culture or from plates incubated in an anaerobic jar. All are large gram-positive rods, non-encapsulated with the exception of *Cl. welchii*, and motile by means of peritrichous flagella with the exception of *Cl. welchii*. Spores are usually of a greater diameter than the vegetative cells, and the spore-containing cells are spindle- or club-shaped. The spores of the tetanus bacillus are round and terminal, and those of the other bacilli oval and subterminal. Two general physiological types of anaerobic bacilli may be distinguished, the one predominantly fermentative or saccharolytic and the other predominantly proteolytic. These and other characteristics are summarized in the accompanying table.

Large amounts of volatile organic acids are produced in the fermentation of carbohydrates by these bacilli; they differ in this respect from the other pathogenic bacteria which produce non-volatile acid, *i.e.*, lactic acid, for

the most part. Amino acids are vigorously attacked by the obligate anaer-
obes; some are "fermented" to organic acids,[2] while others are mutually
oxidized and reduced, a process that has been discussed elsewhere (p. 106).

TETANUS (CLOSTRIDIUM TETANI)

Tetanus is a disease of man and animals characterized by spasms of the
voluntary muscles. The spasms are often most marked in the muscles of
the jaw and neck, hence the name "lockjaw." The tetanus bacillus was first
described in 1884 by Nicolaier, who observed it in the pus taken from mice
and other animals that had died after subcutaneous inoculation with small
quantities of soil. Kitasato isolated the microorganism in pure culture in 1889
and demonstrated its causal significance. He also proved the inability of the
tetanus bacillus to invade the blood stream and showed the disease to be

Fig. 128. *Clostridium tetani* in pure culture. Young, actively growing culture show-
ing beginning spore formation. Note the refractile, unstained spores, the drumstick
appearance when these are attached to the cells, and the tendency of the vegetative cells
to remain attached end to end. Fuchsin; × 1150.

an intoxication. In 1890 von Behring and Kitasato laid the basis for anti-
toxic therapy in their discovery of diphtheria and tetanus antitoxins.

Morphology. Individual tetanus bacilli are slender, motile (20 to 30
peritrichous flagella), gram-positive, sporulating rods with rounded ends.
Their common dimensions are 0.3 to 0.5 μ in width and 2 to 5 μ in length,
but vegetative filaments of much greater length occur. The shorter forms
are usually straight; filaments tend to curve in an undulating manner. Short
chains of rods may occur. The spore is spherical and terminal and larger in
diameter than the vegetative cell; spore-containing cells have a characteristic
drumstick appearance (Fig. 128). Isolated colonies in deep dextrose agar have
a woolly appearance and may either be flocculent or have an opaque center.
Surface colonies are flat, rhizoid, or even feathery, and frequently exceed 1

[2] Such as the conversion of glutamic acid to butyric and acetic acids, carbon dioxide,
ammonia and hydrogen. *Cf.* Barker: Jour. Bact., 1938, 36:322.

mm. in diameter. Later the centers may become slightly raised. Colonies on blood agar show hemolysis.

Physiology. The tetanus bacillus develops in plain or dextrose broth and in brain, meat, agar and gelatin media from which the air has been expelled by heating and excluded by some form of seal. If the depth of media is adequate, say 10 to 12 cm., no special seal is required, especially for the more viscous media. Growth occurs between 14° and 43° C.; the optimum temperature is 37° C.

The growth of *Cl. tetani* is influenced greatly by the presence of associated microorganisms. In sugar-free media it may be grown in mixed cultures upon the surface of culture media in contact with air through the absorption of oxygen by the associated aerobes. But in dextrose broth the growth of the tetanus bacillus in mixed culture is likely to be inhibited by acid formation due to the associated bacteria. Sugar-free media, such as meat infusion peptone broth or deep meat or brain media, are therefore preferable for initial culture from contaminated material. Pure cultures may be isolated from these initial cultures by deep agar or surface culture methods.[3] It has been supposed that an initial heating of the contaminated material simplifies isolation of the tetanus bacillus by destroying the vegetative cells of other microorganisms that may be present; this method may, however, leave the spores of the tetanus bacillus still badly mixed with those of other aerobic and anaerobic bacteria, some of which may be more resistant than the tetanus spores.

In pure culture, dextrose stimulates growth as in the case of the other anaerobes, although dextrose and other carbohydrates are not fermented by *Cl. tetani*. Sporulation is not inhibited by carbohydrates, however, as with the fermentative anaerobes; on the contrary, sporulation of the tetanus bacillus is accelerated in dextrose broth.

Growth in gelatin stab is slow at 22° C., but in ten days or so the characteristic inverted fir-tree type of growth is apparent. Incubated at 37° C. for two to three days, gelatin cultures usually fail to stiffen in the refrigerator. Coagulated proteins, such as blood serum and egg white, are very slowly liquefied. Deep brain and meat media may be slightly softened, but never digested fully; after several weeks a slight darkening occurs near the surface exposed to air. The tetanus bacillus is, therefore, only weakly proteolytic. Amino acids are, however, utilized readily but not by paired oxidation-reduction; glutamic acid, aspartic acid and serine, for example, are attacked directly with the formation of CO_2, NH_3 and acetic and butyric acids.[4] Both amino acids and carbon compounds are readily dehydrogenated.[5] In litmus milk there is reduction of the indicator and sometimes a slight precipitation of the casein. Nitrates are not reduced to nitrites, but hydrogen sulfide and indol are produced.

The tetanus bacillus has been grown in synthetic media and its growth requirements are relatively complex, including the amino acids arginine, histidine, tyrosine, valine, leucine, isoleucine and tryptophane; the bacterial

[3] *Cf.* Reed and Orr: Proc. Soc. Exp. Biol. Med., 1941, *48*:535; War Med., 1941, *1*:493.
[4] Clifton: Jour. Bact., 1942, 44:179.
[5] Pickett: Jour. Biol. Chem., 1943, *151*:203.

vitamins riboflavin, pantothenic acid, thiamine, folic acid, biotin, pyridoxine and nicotinic acid; the purines adenine and uracil; and oleic acid.[6] The formation of toxin is inhibited by iron and best yields are secured when as much iron as possible is taken out of the medium, though undoubtedly very small amounts are required. These and other factors influencing toxin formation have been studied in detail by Mueller and Miller[7]; a beef infusion medium containing hog stomach autolysate or tryptic digest of casein, together with glucose, carbonate, potassium, magnesium and phosphate, allows the production of toxin to high titer.

Spore formation begins in about two days at 37° C. and in eight or ten days at room temperature. The spores are highly resistant and when protected from light and heat remain viable for years. Theobald Smith found a number of strains that resisted steaming at 100° C. for forty to sixty minutes. Five per cent phenol is said to destroy tetanus spores in ten to twelve hours; the addition of 0.5 per cent hydrochloric acid may reduce the time to two hours.

Antigenic Structure. The tetanus bacillus is antigenically heterogeneous, and a number of types, designated by Roman numerals, have been described.[8] At present ten in all are known.[9] Both somatic and flagellar antigens appear to be involved, the former of a group and the latter of a specific nature. Type VI organisms are not flagellated and lack type-specific antigen, and Types II, IV, V and IX have a common O antigen which results in higher titer cross reactions among these than among the other types.[10] Types I and III have been found most commonly in the United States, England and France, Type V in China. The toxin formed by these types is immunologically identical.

Tetanus Toxin. As indicated elsewhere, the potent soluble toxin produced by the tetanus bacillus is of dual nature; *tetanospasmin* is the portion that affects the nervous tissue and *tetanolysin* is a hemolysin. The former is by far the more important; there is no indication that tetanolysin has any significance in connection with the ordinary symptom-complex. Liquid cultures are usually highly toxic; 5×10^{-6} ml. may be fatal to a mouse.

The toxin is filterable and may be freed from bacteria by filtration through Berkefeld, Chamberland and similar filters. In aqueous solution the toxin is highly unstable to heat and light and must be stored in a cold dark place. It is apparently protein in nature, or at least has not as yet been separated from protein, and is destroyed by proteolytic enzymes and hence is ineffective when given by mouth. It may be precipitated with ammonium sulfate and in the dry state retains its potency for a long time. It is an excellent antigen and gives rise to high titer antitoxic sera.

As indicated above, tetanus toxin is one of the most potent poisons known, and its toxicity far exceeds that of alkaloids and other substances generally regarded as highly poisonous. It has been prepared in crystalline form by

[6] Mueller *et al.:* Jour. Bact., 1942, 43:763; *ibid.,* 1943, 46:559, 563.

[7] *Cf.* Mueller and Miller: Jour. Immunol., 1947, 56:143.

[8] *Cf.* Gunnison: Jour. Immunol., 1937, 32:63.

[9] The tenth type has been described by MacLennon: Brit. Jour. Exp. Path., 1939, 20:371.

[10] Gunnison: Jour. Immunol., 1947, 57:67.

precipitation with methanol in the cold by Pillemer, Wittler and Grossberg.[11] These preparations contained 5 to 7.5 $\times$ 10^7 LD_{50} doses for mice per mg. of nitrogen, and tetanus was produced in mice with as little as 0.000013 γ of the crystalline toxin. Usually an incubation period intervenes between inoculation and appearance of symptoms which cannot be reduced to less than eight hours with the usual toxic filtrate and beyond that point the incubation period is inversely related to the amount of toxin injected. With crystalline material doses of as much as 500,000 MLD are possible in the mouse, and these large amounts produce symptoms in thirty minutes and death in an hour.[12]

The Dissemination of Tetanus Toxin in the Body. There are two views concerning the pathway by which tetanus toxin reaches the central nervous system. According to the experimental evidence of Meyer and Ransom,[13] tetanus toxin is absorbed by the end-organs of the motor nerves and travels to the ganglion cells of the central nervous system, not by way of the blood or lymph channels, but along the axis-cylinders of the peripheral nerves. The time consumed in this passage represents the larger part of the incubation period. The toxin may circulate for a time in the blood, but the only path to the central nervous system lies along the axis-cylinders of the motor nerve tract. Intravenous injection of toxin into experimental animals causes only general tetanus, according to Ransom's later reiteration of this view.[14] A cut nerve takes up the toxin very slowly and a degenerate nerve not at all. Section of the spinal cord prevents the toxin from reaching the brain. Meyer and Ransom believed that the spinal ganglion of the sensory nerve presents a barrier to the advance of the toxin, and that for this reason sensory nerves are unable to conduct the toxin. The remarkable excitation of the motor cells of the spinal cord that is observed in tetanus is not accompanied by charateristic lesions.

Although long generally accepted, this view has been challenged in recent years by Abel and his co-workers,[15] who propose the following theory as in accord with their experimental findings: The toxin exhibits both a central and a peripheral action, each of which may be demonstrated independently of the other. The central effect, which is characterized by reflex motor convulsions, is due to the poisoning of the motor nerve cells of the spinal cord, medulla and pons; the peripheral effect, recognized as the unremitting rigidity of voluntary muscles, results from the fixation of the toxin upon the motor end-organs. Following subcutaneous or intramuscular injection the toxin is absorbed by the lymphatics and distributed to the central nervous system by way of the arterial circulation. Normal tonicity of the motor end-organs seems to be essential to the development of tetanic rigidity; neurotomy results in an immediate, sharp depression of the tonicity of the myoneural junctions, hence they are not responsive to the influence of tetanus toxin.

Abel's views have not been generally accepted in spite of their support by convincing experimental evidence which cannot be reviewed here. Other

[11] Pillemer, Wittler and Grossberg: Science, 1946, *103*:615.
[12] Pillemer and Wartman: Jour. Immunol., 1947, *55*:277.
[13] Meyer and Ransom: Arch. f. Exp. Path. u. Pharm., 1903, *49*:369.
[14] Ransom: Lancet, 1917, *ii*:928.
[15] In a series of publications beginning in 1934. Reference may be made to the ninth paper by Abel, Warfield and Chalian: Bull. Johns Hopkins Hosp., 1938, *63*:373.

experimental data have been presented in support of the theory of the axis-cylinder pathway,[16] and as yet no conclusion may be drawn.

Tetanus toxin possesses a strong affinity for the cells of the central nervous system, as evidenced by the now classic experiments of Wassermann and Takaki.[17] A mixture of toxin and brain substance can be injected into an animal without producing any toxic effect, the toxin apparently entering into a firm combination with some ingredient of the nerve substance. Not only the central nerve cells, but to some extent other tissue cells, are able to bind tetanus toxin. Subcutaneous inoculation is less likely to result fatally than direct inoculation into nerve tissues because some of the toxin is bound and prevented from reaching the highly sensitive nerve cells.

Pathogenicity. Tetanus is essentially an intoxication. The bacilli set up a localized infection and the toxin formed there is disseminated through the body and gives rise to the symptom-complex characteristic of the disease. Bacillemia may occur very rarely, however, and has been produced experimentally. The bacilli generally gain entrance to the tissues by means of a deep, dirty wound which may be relatively small, so small sometimes as to escape serious attention. The widespread occurrence of the tetanus bacillus would seem out of harmony with the relative infrequency of tetanus infection but, it may be noted, mere introduction of the bacillus into the body is not sufficient to produce the disease; the microorganisms must find favorable conditions for proliferation at the site of penetration. Experimentally, pure cultures of vegetative cells or spores that have been freed from toxin cannot germinate in uninjured tissues, but simultaneous inoculation with common saprophytes, such as *Bacterium prodigiosum,* or with irritant chemicals, such as calcium salts or lactic acid, enables the bacilli to grow and form toxin. As indicated elsewhere (p. 74), a sufficiently low oxidation-reduction potential is necessary for the germination of tetanus spores, and it is not unlikely that the potential of normal tissues is too high to allow germination but is reduced by injury.

Tetanus of the newborn or *tetanus neonatorum* is a consequence of infection of the umbilicus through septic midwifery. It is especially common among the Negroes of the southern states and in other races living under unhygienic conditions.

The tonic spasms characteristic of tetanus usually begin at the site of infection, and the initial symptoms may include headache and stiffness of the neck. The spasms may remain localized in mild infections, but usually they are general and involve the whole somatic muscular system. Postmortem findings are insignificant; other than a moderate congestion, the organs show no pathological changes and the initial lesion may, of course, be inapparent or small.

The incubation period of tetanus is variable and may range from two to fifty days. The case fatality is inversely related to the incubation time; it may be as high as 70 to 80 per cent or as low as 15 to 20 per cent. Death, if it occurs, follows relatively soon after the appearance of symptoms; the dictum of

[16] Cf. Friedemann, Hollander and Tarlov: Jour. Immunol., 1941, 40:325; Roofe: Science, 1947, 105:180.

[17] Wassermann and Takaki: Ber. klin. Wchnschr., 1898, 35:5.

Hippocrates, "such persons as are seized with tetanus die within four days, or if they pass these they recover," still stands. When the disease has a prolonged incubation period, a less sudden development of symptoms, and consequently more favorable prognosis, it is sometimes termed "chronic."

Lower Animals. Tetanus is not a rare affection in the horse, the symptoms and course of the disease being similar to the disease in man. Cattle, sheep and hogs are less commonly affected. Experimentally, tetanus can be produced in mice and guinea pigs by the inoculation of spores introduced on splinters of wood, and also by injection of toxin. The feeding of animals with tetanus bacilli, spores or toxin is without effect. Tetanus differs from most other infectious diseases in that the diseased animal is not an appreciable factor in the spread of infection. A normal horse apparently may distribute tetanus spores quite as widely and freely as a horse sick with tetanus.

Immunity. As indicated earlier, tetanus toxin is an excellent antigen and high titer antitoxic sera may be prepared. Horses are good producers of antitoxin and are immunized by toxin-antitoxin mixtures followed by toxin alone. In France toxoid is used for the first injections.

The Standardization of Antitoxin. The antiserum is standardized according to the method of Rosenau and Anderson,[18] in which the American immunity unit is defined as "ten times the least quantity of antitetanic serum necessary to save the life of a 350 gm. guinea pig for ninety-six hours against the official test dose of a standard toxin furnished by the Hygienic Laboratory of the Public Health and Marine Hospital Service." The official test dose is about 100 guinea-pig MLD's of a precipitated standard toxin. It may be noted that the L_0 rather than the L_+ endpoint is determined. Thus the tetanus antitoxic unit has slightly more than 10 times the experimental protective power of the diphtheria antitoxic unit. An antitoxic unit strength of 900 per ml. may be attained exceptionally. Practice has been different in other countries, however, and the Permanent Committee on Standardization of the Health Organization of the League of Nations found that 1 German unit, 66 American units and 3750 French units were equivalent. It has been agreed that the international unit shall be one-half of the American unit.[19]

The appearance of multiple zones of precipitation has interfered with the application of the Ramon flocculation method to tetanus toxin, toxoid and antitoxin. Goldie, Parsons and Bowers[20] have found, however, that a specific flocculation zone occurs with refined enzyme-treated antitoxin.

The antitoxin deteriorates with time, of course, the most important factor being the temperature at which it is stored. Amies[21] has found that during storage for one year in the refrigerator no potency is lost, at room temperature the loss is 7 to 9 per cent, and at 37° C. it is 44 to 47 per cent.

The Prophylactic Use of Antitoxin. In veterinary practice, tetanus antitoxin has been used prophylactically with a high degree of success. Vaillard collected the statistics from 1896 to 1906 of eight veterinary surgeons who inoculated

[18] Rosenau and Anderson: U. S. Pub. Health Serv. Bull. No. 43, 1908.
[19] *Cf.* Prausnitz: *Memoranda on the International Standardization of Therapeutic Sera and Bacterial Products.* League of Nations Health Organization. 1929.
[20] Goldie, Parsons and Bowers: Jour. Inf. Dis., 1943, 71:212.
[21] Amies: Brit. Med. Jour., 1941, p. 709.

13,124 animals after operations or accidental wounds, without the occurrence of a single case of tetanus. During the same time two veterinary surgeons alone saw 139 cases of tetanus among animals which did not receive the treatment. The figures of Nocard and Labat added to Vaillard's data cover the cases of 16,917 animals receiving prophylactic injections; among them only one horse had tetanus. In this case the antitoxin was given five days after the wound and the attack was mild.

It is probable that in many cases tetanus is averted in man by the prophylactic use of antitoxin. Even though the disease is not prevented the incubation period is delayed and the disease may be very mild or remain localized. Precise statistical evidence involving comparable series of controls is not, of course, available, but the experience of the first World War was that with increased use of tetanus antitoxin there was not only a lower incidence of the disease but an increase in mild and chronic cases. Passive immunization provides antibody in the circulating blood which combines with toxin and renders it harmless. The avidity of the nervous tissue for tetanus toxin is very great, however, and symptoms may appear in spite of the presence of circulating antibody. Passive immunity is transient, of course, and repeated injections of antitoxin at six- to seven-day intervals must be made as long as danger of infection remains.

The Therapeutic Use of Antitoxin. Tetanus antitoxin appears to have but limited therapeutic value. It is obvious, of course, that symptoms result from damage to the nerve tissue and the administration of antitoxin will neither repair such damage nor displace the more avid nerve tissue already in combination with toxin. Reports on the therapeutic value of antitoxin are conflicting; some workers contend that intrathecal administration either alone or combined with intramuscular injection has a beneficial effect. The records of Cook County Hospital in Chicago, however, show that the therapeutic use of antitoxin has not reduced the mortality from tetanus there.[22]

Active Immunization. In recent years some emphasis has been placed upon active immunization against tetanus, particularly by the French workers. In a summary of the results of twelve years' experience with active immunization of horses and men with formol toxoid, Ramon[23] states that in one cavalry unit in which tetanus was endemic more than 50,000 horses were immunized over a ten-year period and tetanus has practically disappeared, and in a million and a half human beings immunized with toxoid no case of tetanus has occurred.

With the beginning of the second World War, active immunization against tetanus was adopted by the armed services of France, Britain and the United States. Both fluid and alum-precipitated toxoid are used, in this country the former by the Army and the latter by the Navy, and appear to be equally effective though three doses of fluid toxoid are required as against two doses of alum-precipitated toxoid. No international standard exists for tetanus toxoid; the United States Army specified that the toxin contain at least 10,000 guinea pig MLD's per ml. and be detoxified with 0.4 per cent formalin; the final preparation must be atoxic for guinea pigs in 5 ml. amounts, and pigs receiving 1 ml. as an immunizing dose must be able to withstand 10 MLD of toxin at the

[22] Calvin: Jour. Amer. Med. Assn., 1930, 94:1977.
[23] Ramon: Rev. Immunol., 1939, 5:477.

end of six weeks.[24] More rapid methods of assay using mice have been suggested. For immunization three doses of 1 ml. each are given at intervals of three weeks and a stimulating dose one year later. The value of more than two doses is illustrated by Evans[25] who found that the mean antitoxin titer after two inoculations was 0.35 IU (International Units) but with a third inoculation ten months later the mean titer rose to 10 IU and eighteen months later was still 0.37 IU.

In practice this immunization appears to be highly effective. In the period 1942–45 only 12 cases of tetanus occurred in the United States Army, of which 6 were in unimmunized persons,[26] and 4 cases in the United States Navy of which 3 were in unimmunized persons; in contrast a tetanus rate of about 10 per 100,000 wounded prevailed in the Japanese army and navy which did not practice routine immunization. British experience in the Middle East war zone similarly indicated an effective immune response, though in most cases only two doses were given.[27] The success of active immunization, coupled with the low incidence of untoward reactions (reported to be 1 in 10,000 immunizations with improved toxoid), has suggested its more general application in civil life. In France, where active immunization to tetanus has been of more and earlier interest than elsewhere, it has been made compulsory as with diphtheria immunization.[28] In this country considerable interest has attached to combined tetanus and diphtheria immunization of children.

GASEOUS GANGRENE[29]

Gaseous gangrene is a syndrome often following dirty, lacerated wounds, especially those involving fractures. It is a characteristic complication of war wounds, and present knowledge of this affection was largely developed during the first World War. But this disease is by no means so rare in civil life as was

OUTSTANDING PATHOLOGICAL CHANGES IN GUINEA PIGS INOCULATED
WITH ANAEROBES OR THEIR TOXINS

(Hall)

	Edema	Emphysema	Congestion	Histolysis
Cl. tetani	–	–	+	–
Cl. septicum	+	+ +	+ + +	+
Cl. welchii	+ +	+ + +	+	+
Cl. novyi	+ + +	–	–	–
Cl. sordellii	+ + +	–	+	–
Cl. histolyticum	–	–	+ +	+ + +
Cl. chauvei	+	+ +	+ + +	+
Cl. botulinum	–	–	+	–

[24] Long: Amer. Jour. Pub. Health., 1943, 33:53.
[25] Evans: Lancet, 1943, ii:316.
[26] Long and Sartwell: Bull. U. S. Army Med. Dept., 1947, 7:371.
[27] Boyd and MacLennon: Lancet, 1942, i:745.
[28] Bull. Office Internat. d'Hyg. Publique, 1940, 32:748.
[29] See the general review by Danielson: Trans. New York Acad. Sci., Ser. II, 1947, 9:297.

formerly thought. The increased number of injuries in automobile accidents is responsible for many cases of gangrene. Men injured about railroad tracks, either employees or vagrants, seem particularly prone to develop gaseous gangrene if not properly and promptly treated. Certain forms of peritonitis, appendicitis, intestinal obstruction, puerperal sepsis and postoperative infections (particularly after laparotomy) are etiologically closely related to it.

In the fulminating form of gaseous gangrene the muscles become filled with gas and with a serosangineous exudate depending for its character upon the associated microorganisms, for this disease is nearly always a mixed infection of aerobes and anaerobes of several species. The accompanying table shows the chief pathological changes in guinea pigs inoculated with various anaerobes or their toxins.

THE FREQUENCY OF OCCURRENCE OF ANAEROBIC BACILLUS SPECIES
IN GASEOUS GANGRENE

Species	Mixed Anaerobic Flora		Pure Anaerobic Flora	
	Cases	Per Cent	Cases	Per Cent
Cl. perfringens (welchii)	91	72	37	74
Cl. sporogenes	34	27	2	4
Cl. edematiens (novyi)	33	26	6	12
Cl. fallax	26	21	3	6
Cl. septicum	12	9.5	1	2
Cl. tetani	11	8.7	—	—
Cl. histolyticum	8	6.3	—	—
Cl. aerofoetidum	5	3.9	1	2
Cl. putrificum	2	1.6	—	—
Cl. bifermentens (sordellii)	2	1.6	—	—
Cl. tertium	1	0.8	—	—
Bacillus II of Ghon and Sachs	1	0.8	—	—

Weinberg and Séguin[30] studied 126 cases of gangrenous wound infection other than tetanus. Of these, 35 were phlegmonous and 6 of them yielded only aerobic bacteria; the remainder contained anaerobic bacilli. Thus aerobes are apparently able to produce gaseous phlegmons, but the number of such cases is small; gaseous infections are predominantly anaerobic and the role of aerobes is, as a rule, only accessory. Of the 12 cases of anaerobic infection, 90 contained both aerobes and anaerobes; 70 contained more than 1 anaerobe and 50 but a single anaerobic species. The frequency with which the various anaerobic species were found is summarized in the accompanying table. Similar data collected during the World War II by MacLennon[31] showed somewhat different proportions but the same general trend, a predominance of *Cl. welchii, Cl. novyi* and *Cl. septicum.*

Clearly, then, the bacteriology of gaseous gangrene is complicated and

[30] Weinberg and Séguin: *La Gangrène Gazuse.* Masson et Cie., Paris. 1918.
[31] MacLennon: Lancet, 1943, *i*:63, 94, 123; *ibid.,* 1944, *ii*:203.

while, as stated by Weinberg and Séguin, there is in effect a typical form of gaseous gangrene, (*a*) it is not always produced by the same microorganism; (*b*) it is frequently caused by several associated agents; (*c*) it is often the complex result of the combined action of these principal anaerobic bacilli with various other bacteria which play an indeterminate accessory role.

The more important of the anaerobic bacilli associated with gaseous gangrene are discussed in the following sections.

1. THE VIBRION SEPTIQUE, CLOSTRIDIUM SEPTICUM

In 1877 and 1881 Pasteur, while studying anthrax, produced a septicemia in rabbits and guinea pigs by the inoculation of putrid blood from a cow. The affection could be communicated from individual to individual, and a sporulating, motile, rod-shaped anaerobe, considered by him "one of the vibrions of putrefaction" (the actively motile bacilli sometimes appear to be

Fig. 129. *Clostridium septicum* from pure culture. The tendency to form elongated vegetative cells is apparent. Fuchsin; × 1050.

curved), was regarded as the cause of the septicemia and named "vibrion septique."

In 1881 Koch described the pathological effects of a microorganism which he declared identical with the vibrion septique of Pasteur. But this bacterium failed to produce a septicemia in guinea pigs, and since its pathogenic effects were limited largely to the site of inoculation, Koch designated it "the bacillus of malignant edema."

Neither Pasteur's nor Koch's description would suffice now to identify with certainty the microorganisms in question. Fortunately, the original strain of Pasteur's "vibrion septique" has been maintained in France, so that its outstanding characteristics are well known. The lack of any such legacy from Koch in Germany, due to his failure to recover cultures, has led to an all but interminable discussion as to the properties of the "true bacillus of malignant edema." The vibrion septique is now generally known as *Cl. septicum*.

Morphology. The vibrion septique is a gram-positive, sporulating, spindle-shaped rod, or filament, and in young cultures motile, with many

peritrichous flagella. The ends are slightly rounded and the spores, which are oval, are usually median and swell the vegetative cell into a clostridium previous to their release. Spores are formed only in media not containing fermentable carbohydrate in excess. The long chains and filaments of these microorganisms which occur on the visceral surfaces of infected guinea pigs are of high differential value. Capsules have never been observed. Deep colonies in 1 per cent agar are transparent or semitransparent. Hemolysis occurs on blood agar.

Physiology. *Cl. septicum* is a strict anaerobe and develops readily in deep brain or tissue media, producing gas rather abundantly. These media are not discolored even in the presence of metallic iron. Gelatin is liquefied, but coagulated serum and other proteins are not digested or blackened. Hydrogen sulfide is produced but indol is not. Dextrose, levulose, galactose, maltose, lactose and salicin are fermented; media not containing one of these sugars support only slight growth. Sucrose, inulin, mannitol and dulcitol are not fermented. The fermentation of salicin and non-fermention of sucrose allow the biochemical differentiation of *Cl. septicum* and *Cl. chauvei*, for the latter does not ferment salicin but does ferment sucrose.

Antigenic Structure and Toxin. Strains of *Cl. septicum* are immunologically related but distinct. Four groups have been distinguished on the basis of the somatic antigen, and subdivisions may be made by the flagellar antigen. These bacilli are immunologically related to *Cl. chauvei*. The toxin formed, however, is specific, but a relatively weak lethal agent. The MLD for mice is about 0.005 ml. Bernheimer[32] has prepared a dialyzable medium containing casein hydrolysate, cystine, tryptophane, glutamine, biotin, thiamine, nicotinic acid, pyridoxine, glucose, thioglycollic acid and inorganic salts which supports the growth of some strains with the formation of 400 to 500 mouse LD_{50} doses of toxin per ml. When injected into animals it produces a gelatinous edema and some local necrosis of the tissues. According to Kellaway, Reid and Trethewie[33] the toxin has a specific cardiac action in the cat and rabbit, producing a fall in systemic and a rise in venous blood pressure, in the cat a specific constriction in the pulmonary and coronary circulations, with edema of the lungs and loss of fluid from the circulation.

Pathogenicity. *Cl. septicum* does not occur in gaseous gangrene of man as frequently as some of the other anaerobic bacilli but has been found in such affections both alone and in mixed cultures. It has been recovered from gaseous infections in cattle and may be one of several microorganisms responsible for blackleg, usually considered a specific disease due to *Cl. chauvei*. It has also been found in gaseous infections of hogs and other domestic animals. Experimentally the vibrion septique is strikingly pathogenic for chickens, pigeons, rabbits, guinea pigs, rats and mice. In such animals the bacteria develop rapidly, producing gas and a reddish, serous edema. They invade the adjacent tissues and the circulation, producing a septicemia which is usually fatal within twenty-four to forty-eight hours; sublethal doses do not produce any reaction. Impression smears from the tissues, and especially from the liver,

[32] Bernheimer: Jour. Exp. Med., 1944, 80:321.
[33] Kellaway, Reid and Trethewie: Australian Jour. Exp. Biol. Med. Sci., 1941, 19:297.

usually show elongated filaments or chains as contrasted with the single bacilli found in animals killed with *Cl. chauvei.*

Antitoxic sera which are prophylactic and, to some degree, curative may be prepared by injection of *Cl. septicum* toxin into horses. The antisera do not have the high antitoxin content that is found in antitetanic sera. Polyvalent commercial sera for prophylactic and therapeutic use in wound infections often contain antibodies to *Cl. septicum.*

2. CLOSTRIDIUM WELCHII (CLOSTRIDIUM PERFRINGENS)

Clostridium welchii was first cultivated by Achalme in 1891 and supposed by him to be the cause of articular rheumatism. In 1892 Welch and Nuttall isolated this bacillus from the foamy organs of a cadaver and called it *Bacillus aerogenes capsulatus.* Found by Fränkel the following year, it was designated

Fig. 130. *Clostridium welchii* from pure culture. Note the relatively smaller size of these bacteria and the central spores. Fuchsin; × 1050.

Bacillus phlegmonis emphysematosae, and in 1897 Veillon and Zuber called it *Bacillus perfringens.* Sometimes called Fränkel's bacillus in Germany, *Cl. perfringens* in France and *Cl. welchii* in English-speaking countries, it is designated *Cl. perfringens* by Bergey.

Morphology. *Cl. welchii* is a plump, non-motile, gram-positive rod of variable length, occurring in chains and singly. Capsules are usually present in preparations made from the organs or body fluids. Spores are formed sparingly and only in the absence of fermentable carbohydrates; they are centrally located, rarely subterminal, and do not swell the vegetative cell in which they are formed. Isolated colonies in deep agar are compact, opaque, white or grayish white biconvex disks. On blood agar the round, smooth, opaque, entire-edged colonies are relatively large, 2 to 5 mm. in diameter, and surrounded by a zone of hemolysis.

Welch's bacillus is a strict anaerobe and grows readily in deep brain, meat infusion broth, agar and gelatin media. Its growth in sugar-free media is

greatly restricted. Optimum conditions for growth are provided by media containing fermentable carbohydrates, but such cultures are often short-lived because of the lack of spore formation and the destructive action of the formed acids on the vegetative cells. Brain and meat media are not blackened normally, but the presence of metallic iron produces a distinct discoloration. Gelatin is liquefied but coagulated serum or egg is not digested. Hydrogen sulfide is produced, indol is usually said to be negative but its formation is uncertain.

Nutritive requirements are complex, and semisynthetic media which support growth include casein hydrolysate or 19 amino acids together with pantothenic acid, thiamine, nicotinic acid, riboflavin, biotin, folic acid, pyridoxine, adenine, guanine, uracil, inorganic salts including those of manganese and iron, together with glucose.[34] The production of a toxin (see below) requires two additional substances, one found in enzymatic digests of certain proteins, and the other an alcohol-soluble constituent of pancreas; glyceryl phosphocholine appears to be in part responsible for the activity of the latter.[35]

Acid and gas are produced in glucose, maltose, lactose and sucrose; neither mannitol nor salicin is fermented; some strains ferment inulin and some glycerol. Broth cultures containing fermentable sugars become markedly turbid with abundant gas formation, and many cultures, possibly all at certain stages, become stringy and viscid. Milk is fermented with a characteristic "stormy" evolution of gas, followed by coagulation of the casein due to acid formation, and the curd is shortly torn to shreds by the continued evolution of gas within. The curd is not digested. This "typical" reaction is considerably modified by incomplete anaerobiosis; gas production may be slow and the solid curd is torn only slightly if at all. Under optimum conditions 3.8 times the volume of the milk may be evolved as gas; hydrogen predominates during the early stages of the fermentation and carbon dioxide in the later stages.

Types. Although *Cl. welchii* strains from gaseous gangrene form the same toxin, they are not immunologically homogeneous. Henderson[36] has observed that the Wilsdon types (see below) are homogeneous with respect to heat-stable antigen with the exception of Type D in which he found six kinds of antigens in thirteen strains. Rodwell[37] has shown that a number of subtypes of each of the Wilsdon types may be distinguished by agglutination and precipitin tests and there are some cross reactions between types. The common antigen appears to be a capsular polysaccharide; this substance has been prepared by Svec and McCoy.[38] As yet, however, serological identification of *Cl. welchii* is not practical. Four biochemical types have been suggested on the basis of differences in the fermentation of glycerol and inulin, but these fermentative differences do not appear to be correlated with other variable characteristics.

Toxigenic bacilli closely resembling *Cl. welchii* and having immunologically related toxins have been isolated from lower animals. These are the lamb dysentery bacillus (*Bacillus agni*), a bacillus causing a disease of sheep called

[34] Boyd, Logan and Tytell: Jour. Biol. Chem., 1947, *167*:879.
[35] Adams, Hendee and Pappenheimer: Jour. Exp. Med., 1947, 85:701.
[36] Henderson: Jour. Hyg., 1940, 40:501.
[37] Rodwell: Australian Vet. Jour., 1941, *17*:58
[38] Svec and McCoy: Jour. Bact., 1944, 48:31.

"struck" (*Bacillus paludis*), and a bacillus responsible for an enterotoxemia of sheep (*Bacillus ovitoxicus*). Wilsdon[39] has proposed that these be designated *Cl. welchii* but that four types be distinguished: *Cl. welchii* Type A—the gaseous-gangrene bacillus; *Cl. welchii* Type B—the lamb-dysentery bacillus; *Cl. welchii* Type C—the "struck" bacillus; and *Cl. welchii* Type D—the bacillus of enterotoxemia of sheep. Unless otherwise indicated, however, the name *Cl. welchii* always refers to the gas-gangrene bacillus, or Type A.

Toxins. It has been shown that, as a group, these bacilli form seven immunologically distinct toxins and that the observed interrelationships of the toxins formed by the various types are attributable to the sharing of one or more of these components[40] The effects produced by these toxins are as follows:

The α toxin is hemolytic, lethal to mice on intravenous injection, and produces necrosis on intradermal injection into guinea pigs and rabbits. Termed by Wilsdon the W factor and designated by some workers as the ζ toxin.

The β toxin is not hemolytic, mice injected intravenously develop spasmodic twitchings and die almost immediately, and it produces skin necrosis in guinea pigs and rabbits. Termed by Wilsdon the Z factor.

The γ toxin is not hemolytic, does not produce skin necrosis in guinea pigs, and is lethal to mice.

The δ toxin is hemolytic but is not lethal to mice and does not produce skin necrosis.

The ϵ toxin is not hemolytic, but produces skin necrosis in guinea pigs and rabbits and is lethal to mice. Termed by Wilsdon the X factor.

The θ toxin is hemolytic and lethal and probably produces necrosis in high concentrations. It is oxygen-labile and thermolabile and very similar in properties and immunological specificity, though not identical with, streptolysin O. It is the same as Prigge's toxin.

The η toxin has lethal activity only. It has been found in only one strain (Lechien) of Type A as yet examined and its occurrence or absence in the other types is not definitely established.

The optimal conditions of pH, incubation time and composition of the medium differ from one toxin to another, and a type producing more than one toxin will produce only those for which the conditions are optimal. The distribution among the Wilsdon types of the ability to form these toxins is indicated in the accompanying table. The terminology of the toxins has been somewhat confused owing to differences in the terms used by various workers; that used here has been generally agreed upon.

Of these the α toxin has been of greatest interest since it is associated with the virulence of the bacilli. It was observed independently by Nagler[41] and by Seiffert[42] that an opalescence is produced in human serum by the addition of filtrate containing the α toxin. This is known as the *Nagler reaction*. On the assumption that the opalescence is a result of splitting lipoprotein, it was shown by Macfarlane, Oakley and Anderson[43] that incubation of such filtrate with a saline extract of egg yolk, "lecithovitellin," resulted in the separation of a curd of fat. It was then shown by Macfarlane and Knight[44] that an enzymatic split-

[39] Wilsdon: Inst. Animal Path., Cambridge, 2nd report of the Director, 1931, p. 53.
[40] See the review by Oakley: Bull. Hyg., 1943, 18:781.
[41] Nagler: Brit. Jour. Exp. Path., 1939, 20:473.
[42] Seiffert: Ztschr. f. Immunitätsf., 1939, 96:515.
[43] Macfarlane, Oakley and Anderson: Jour. Path. Bact., 1941, 52:99.
[44] Macfarlane and Knight: Biochem. Jour., 1941, 35:882.

ting of free lecithin into phosphocholine and stearyloleylglyceride occurs, and the toxin appears to be a lecithinase. Since phosphocholine is water-soluble, it has been suggested that the α toxin may be estimated by measurement of the liberation of soluble phosphorus under standard conditions.

In addition to the α toxin and θ toxin noted above, *Cl. welchii* forms a collagenase which has been designated the κ toxin which breaks down muscle by dissolution of its collagen and reticulin structure[45]; the part it plays in the pathology of the infection is not clear. It does not appear to be as important as the α toxin, however, for while antiserum to the α toxin alone is protective, anticollagenase alone is not.[46]

TOXINS FORMED BY THE WILSDON TYPES OF CL. WELCHII

Type	Toxins						
	α	β	γ	δ	ϵ	θ	η
Cl. welchii Type A (bacillus of gas gangrene)............	+	−	−	−	−	+	+
Cl. welchii Type B (*Bacillus agni*).....................	+	+	+	±	−	+?	?
Cl. welchii Type C (*Bacillus paludis*)..................	+	+	+	+	−	+?	?
Cl. welchii Type D (*Bacillus ovitoxicus*)................	+	−	−	−	+	+?	?

In general the toxins formed by *Cl. welchii* appear to account in very large part for the observed histopathology. Robb-Smith,[47] for example, has compared that in naturally occurring infection in man, in experimentally infected animals and in normal human muscle exposed to the action of filtrates *in vitro* and found the histopathological changes substantially the same in all three.

There appears to be no simple method of typing *Cl. welchii* and the presence or absence of the various toxins must be demonstrated. The differentiation of the toxins suggested by Wilsdon[39] is relatively simple but too crude for most purposes. A precise method of determining the toxic components of a filtrate using monospecific antisera is outlined by Oakley.[40]

Pathogenicity for Man. *Cl. welchii* is, perhaps, the most important cause of gaseous gangrene and is found either alone or mixed with other anaerobes in the majority of cases of this disease. Tissue injury is a usual, perhaps essential, preliminary to infection, but once the bacilli are established they invade the surrounding tissue rapidly. They apparently travel along the interstitial tissue of the muscle and are often found beyond the gangrenous area. The large amount of hyaluronidase produced would seem to be related

[45] Oakley, Warrack and van Heyningen: Jour. Path. Bact., 1946, 58:229.
[46] Evans: Brit. Jour. Exp. Path., 1947, 28:24.
[47] Robb-Smith: Lancet, 1945, ii:362.

to this rapid spread, but a number of studies have indicated that there is little or no relation between the invasiveness of *Cl. welchii* and at least *in vitro* titers of hyaluronidase.

Although most commonly found in gangrene, *Cl. welchii* has also been observed in closed abscesses in uterine infections, and in infections of the gastro-intestinal, genito-urinary and biliary tracts. It has been isolated from the blood during life, but septicemia in man is much less common than in experimental animals, although blood invasion occurs frequently in man during the agonal period or immediately following death. Study of the "foamy organs" sometimes observed at autopsy has shown that the presence of gas in the internal organs shortly after death is often attributable to an invasion by this microorganism.

Cl. welchii is a normal inhabitant of the human intestine and is constantly present in small numbers; in fact, it has been used to a certain extent in Europe, as an indicator of fecal pollution of water (p. 253). The toxemia of acute intestinal obstruction has been attributed by some to the proliferation of *Cl. welchii* in the bowel followed by absorption of formed toxin, but it is now clear that such a relationship does not exist. This bacterium is, however, found with some frequency in gangrenous appendicitis and it has been reported that antitoxin is of value in the treatment of perforative appendicitis.

Since infection with *Cl. welchii* is frequently characterized by gross blood destruction, jaundice and anemia, a possible relationship between this bacterium and various anemias has been of some interest. A pernicious and fatal anemia may be produced in experimental animals by intratibial inoculation of culture or a temporary but severe anemia by inoculation of filtrate. Both natural and experimental infections, therefore, lead to the development of a severe anemia which is probably due to the continuous release of the hemolytic toxin.

Pathogenicity for Animals. Natural infections in lower animals with *Cl. welchii* types have been referred to above. The occurrence of the gas-gangrene bacillus, however, is rare; local abscesses have been observed in dogs and rabbits following injury. Experimentally certain strains are pathogenic for guinea pigs, pigeons and mice, less so for rabbits. If a rabbit or guinea pig is killed a few minutes after intravenous injection of *Cl. welchii* and the body incubated at 37° C., gas is produced in a few hours through the body and the phenomenon of "foamy liver" reproduced. This phenomenon is not strictly specific for *Cl. welchii*; it may be produced by similar inoculations with several other anerobes, though the results are less striking. The pigeon is susceptible and is used for the standardization of toxin and antitoxin.

The classic *welchii* toxin, the α toxin, is not a powerful one; the MLD for a mouse is usually about 0.25 ml. of liquid culture. Antitoxins may be produced, however, which have both prophylactic and therapeutic value, and *welchii* is included in polyvalent antitoxic sera for gas gangrene. Antitoxin to the α toxin appears to be far more important than that to the θ toxin.[48] It may be noted that it is difficult to develop agglutinins for *Cl. welchii* and an antibacterial immunity does not protect against infection.

[48] Evans: Brit. Jour. Exp. Path., 1943, 24:81.

3. CLOSTRIDIUM NOVYI (CLOSTRIDIUM OEDEMATIENS)

The third important anaerobe in gaseous gangrene was probably first discovered by Novy in 1894 in a study of "malignant edema" in guinea pigs, and was designated *"Bacillus oedematis maligni Nr. II."* It was named *Bacillus novyi* by Migula in 1900. In 1915 Weinberg and Séguin isolated several strains of this bacillus, but first regarded it as a new species and named it *Bacillus oedematiens.* The French name has been used by European workers although the bacillus is properly known as *Clostridium novyi.*

Cl. novyi is noteworthy not only for its importance in gaseous gangrene, but also because of its strong, soluble exotoxin, which compares in potency with
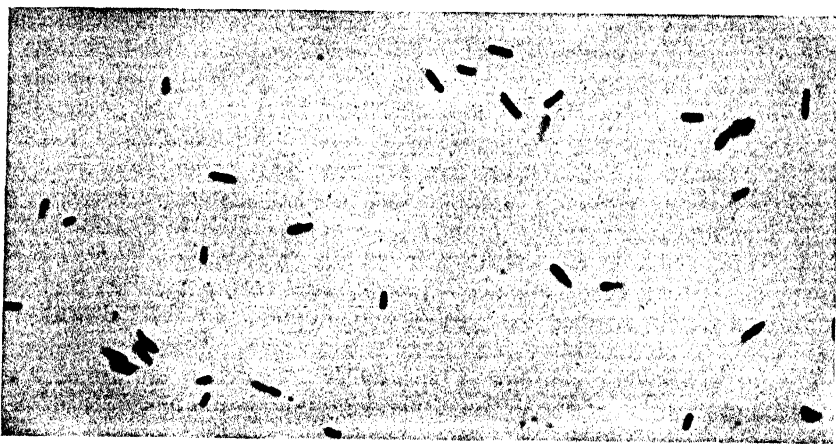
Fig. 131. *Clostridium novyi* from pure culture. The slight tendency to curvature is apparent in some of the vegetative cells. Note the subterminal spores and the absence of free spores in large numbers. Fuchsin; × 1050.

the toxin of the diphtheria and tetanus bacilli, and for which an equally powerful antitoxin can be produced.

Morphology. Novy's bacillus is a large, relatively thick rod, 2.5 to 10 μ in length and 0.8 to 1 μ in breadth, and occurs singly and in chains. In cultures navicular and curved forms are found; in animal transudates the shorter form predominates. Its numerous spiral flagella, which often become tangled in "bouquets," have been emphasized in nearly all the published descriptions. The rod is non-motile under ordinary conditions of examination, for movement is markedly inhibited in the presence of air. Subterminal spores are produced but sparsely as a rule and best in nonfermentable media. The bacillus is gram-positive.

Young colonies in deep dextrose agar have a yellowish, opaque, irregular center surrounded by a delicate corona of short filaments. Later the colony clears, the center becomes cloudy, and is surrounded in forty-eight hours with a corona of tangled filaments. Surface colonies are extremely delicate, flattened, transparent, bluish gray, with irregular contours, and there is slight hemolysis on blood agar.

Physiology. *Cl. novyi* is a strict anaerobe, and grows well at 37° C. in

ordinary media and especially abundantly in the presence of a fermentable sugar. Meat and brain are not darkened; the former may be turned slightly pink or bleached. Gelatin is liquefied, but coagulated serum and egg are not digested. McLeod and Gordon[49] have reported that *Cl. novyi* may be distinguished from the other sporulating anaerobes by culture on blood agar containing benzidine; the colonies are blackened due to the presence of hydrogen peroxide. Nitrates are not reduced and indol is not formed, but hydrogen sulfide is produced. Acid is produced very slowly in litmus milk; after ten to thirty days' incubation a fine flocculent clot appears which is not digested. Dextrose is fermented but lactose is not; the latter property serves to differentiate *Cl. novyi* from *Cl. septicum* and *Cl. chauvei*. There is not complete agreement as to the other fermentation reactions.

Antigenic Structure and Toxin. Three immunologic types of *Cl. novyi* have been defined by Scott, Turner and Vawter[50] and designated Type A, Type B and Type C.

The toxin formed by this organism is the most potent of the gaseous gangrene bacilli toxins; in contrast to *Cl. welchii* whose filtrates contain 4 to 5 mouse MLD per ml. the lethal dose of *Cl. novyi* filtrate for the mouse is about 0.005 ml. Lecithinase and hemolysin activity are present, and six components have been identified by Oakley, Warrack and Clarke.[51] These are designated the α, β, γ, δ, ϵ and ζ toxins of which the α toxin is the classic lethal toxin. The distribution of these corresponds to the immunologic types noted above and is illustrated in the accompanying table from Oakley *et al.*

DISTRIBUTION OF TOXINS AMONG *CL. NOVYI* TYPES

Activity of Toxin	Designation	*Cl. novyi* Types		
		Type A	Type B	Type C
Lethal, necrotizing	α	+	+	—
Hemolytic, necrotizing, lecithinase	β	—	+	—
Hemolytic lecithinase	γ	+	—	+?
Oxygen-labile hemolysin	δ	+	—	—
Opalescence in leeithovitellin	ϵ	+	—	—
Hemolysin	ζ	—?	+	—

Pathogenicity for Man. The relatively frequent occurrence of *Cl. novyi* in gas gangrene has been noted earlier. The disease is characteristically a toxemia, although septicemia is not rare. Like *Cl. welchii*, Novy's bacillus is often a terminal invader. In pure infections there is less tissue destruction than with *Cl. welchii* or *Cl. septicum*. The postmortem findings consist mainly in a massive localized edema, with neither the extensive gas production of the former nor the sanguineous necrosis of the latter.

49 McLeod and Gordon: Jour. Path. Bact., 1940, 50:167.
50 Scott, Turner and Vawter: Proc. 12th Int. Vet. Congr., 1934, 168.
51 Oakley, Warrack and Clarke: Jour. Gen. Microbiol., 1947, 1:91.

Pathogenicity for Animals. Natural infections due to *Cl. novyi* have been observed in guinea pigs, cattle, horses and hogs. Guinea pigs, rabbits, rats, mice, cats, sheep, horses and pigeons are susceptible to small doses of culture. Subcutaneous, intramuscular and intravenous inoculations reproduce the disease experimentally. Toxicity and pathogenicity are easily lost, however; Novy's original strain, which still survives in several laboratories, has long since failed to kill experimental animals. This is true also of strains isolated within five to ten years.

The action of the toxin freed from bacteria is very similar to that of whole cultures. Sublethal, subcutaneous doses of toxin or culture produce a peculiar non-hemorrhagic, gelatinous local edema which reaches its maximum in two or three days. It may be followed by small, superficial hemorrhages, after which it is slowly absorbed, leaving a slightly sclerotic scar. Such lesions appear not to form open phlegmons, as in the case of *Cl. welchii* cultures, and may also be contrasted with those of the vibrion septique and *Cl. chauvei,* which, if they appear at all, are always fatal. Washed cultures are harmless.

Antitoxin has been produced in rabbits, sheep and horses by successively increased doses of toxic filtrates. The antitoxin has prophylactic and, to some extent, therapeutic value under experimental conditions, and is now represented in several polyvalent American sera or anaerobic infections.

4. CLOSTRIDIUM HISTOLYTICUM

Among the new species of bacteria discovered by Weinberg and Séguin in war wounds, none is of more interest than *Cl. histolyticum,* so named because

Fig. 132. *Clostridium histolyticum* from pure culture. Note the characteristic short rods with rounded ends and the clostridial subterminal spores. Fuchsin; × 1050.

of its remarkable liquefying action upon living tissues. It may be somewhat more common in gaseous gangrene than indicated earlier. It has also been recovered from soil and human feces and from poisoned arrows.

Morphology. *Cl. histolyticum* is a gram-positive motile rod, 3 to 5 μ long and 0.5 to 0.7 μ wide, that forms subterminal clostridial spores. In smears from lesions it appears generally in the form of single or paired short rods

with rounded ends. The flagella, often more than 20 in number, are peritrichal. Deep agar colonies vary, according to the consistency of the medium, from compact lobulate globules in 2 per cent agar to fluffy semi-transparent or even cottony balls in lower concentrations. Surface colonies are minute, round dewdrops and are hemolytic on blood agar.

Physiology. Originally described as an obligate anaerobe, *Cl. histolyticum* is capable of a delicate transparent growth upon the surface of meat infusion agar and is perhaps best regarded as microaerophilic or as a facultative anaerobe.

This bacillus is actively proteolytic; not only is gelatin liquefied but meat and brain and coagulated serum and egg are digested. In older cultures a precipitate of tyrosine crystals appears. Nitrates are not reduced and indol is not formed. No carbohydrates are known to be fermented in spite of statements to the contrary regarding dextrose. The action on milk is slow, but after several days a soft clot is usually formed and then slowly digested. *Cl. histolyticum* is, clearly, a proteolytic type.

Pathogenicity. Infection with *Cl. histolyticum* alone is probably a rare occurrence; mixed cultures with other anaerobes and aerobes appear to be the rule both in war wounds and in infections observed in horses. Most pure cultures of this bacillus are pathogenic under experimental conditions for rabbits, guinea pigs, mice and rats, but there is considerable difference between strains. Subcutaneous inoculation of 1 or 2 ml. of a twenty-four-hour broth culture generally produces a local tumefaction followed in twenty-four to forty-eight hours by complete sloughing of the overlying skin; then, as a rule, healing slowly occurs. Intramuscular inoculation causes swelling, followed by progressive myolysis. If the gluteus muscle of a guinea pig is selected for the inoculation, it may be entirely denuded from the bone within twenty-four to forty-eight hours. The tissues literally drip away, and in some cases the limb may be disarticulated. Curiously there is often little or no intoxication of the animal, but death usually follows through peritonitis due to perforation of the peritoneum. There is occasionally invasion of the blood stream, but generally septicemia does not occur. There is never any gas formation in such pure infections.

Bacteria-free filtrates have a lytic action which can be demonstrated if sufficiently large quantities (5 ml.) are injected. The most characteristic effect is the formation of a sterile hematoma filled with uncoagulated blood in which the red corpuscles are still intact. According to Pasternack and Bengtson[52] deep intramuscular inoculation produces an edema which disorganizes and separates the tissue, while gross lesions are observed only occasionally following intravenous inoculation. Agglutinating and antitoxic sera have been produced.

5. CLOSTRIDIUM SPOROGENES

Clostridium sporogenes, which in pure culture is a harmless saprophyte, is included here because it is frequently associated with the pathogenic anaerobes in mixed gangrenous infections, very possibly because it is so widely distributed in nature. It has frequently been confused with the pathogenic forms; not only have cultures labeled something else proved to be *sporogenes,* but there has

[52] Pasternack and Bengtson: Pub. Health Repts., 1940, 55:775.

been a tendency to regard "atoxic variants" or "atoxic strains" of pathogens as *Cl. sporogenes.* On the other hand, the presence of "atoxic variants" of pathogenic species is in many cases attributable to mixed cultures containing *sporogenes.* The spores of this microorganism are unusually hardy and invariably survive with those of the pathogens or even after the pathogenic spore-formers are killed in preliminary selective heating.

Morphology. *Cl. sporogenes* is an actively motile, gram-positive, slender rod 3 to 7 μ in length and 0.6 to 0.8 μ in breadth, with rounded ends. The cells occur individually, in pairs, in short chains, and sometimes in filaments. The spores are oval, eccentric to subterminal, and swell the vegetative cell. The flagella are peritrichal. The deep agar colonies have the appearance of woolly balls with a dense, compact center. Surface colonies on blood agar are hemolytic, transparent and usually rhizoid, or ameboid, with a slightly raised center; they appear moist and at first may resemble minute dewdrops.

Fig. 133. *Clostridium sporogenes* from pure culture. Note the close morphological resemblance of this species to the pathogenic forms. Fuchsin; $\times$ 1050.

Cl. sporogenes requires strictly anaerobic conditions for growth and will grow upon all the ordinary media. It has been cultivated in synthetic solutions containing tryptophane, leucine, tyrosine, arginine and phenylalanine, together with an unknown substance termed "sporogenes vitamin."[53] Its optimum temperature is 37° C. but it will grow at temperatures as high as 50° C.

This bacillus is actively proteolytic; it produces blackening and digestion of brain and meat media, coagulated egg and serum. An excess of fermentable sugar delays or inhibits this proteolysis and the presence of metallic iron or certain iron salts accelerates it. Tyrosine crystals are not obvious. Gelatin is liquefied and blackened, hydrogen sulfide is produced, but indol production is doubtful and nitrates are not reduced to nitrites. Reports on sugar fermentations are conflicting. According to Bergey (1939), acid and gas are formed from dextrose, levulose, galactose and maltose, while lactose, sucrose, salicin and inulin are not fermented. Growth on milk is at first slow; in forty-eight to

53 Fildes and Richardson: Brit. Jour. Exp. Path., 1935, 16:326.

seventy-two hours a clot is formed and progressive liquefaction occurs with abundant gas and acid formation until the casein is completely peptonized.

Pathogenicity. There is no authentic record of a natural infection attributable to *Cl. sporogenes* alone. It has been claimed that this bacillus is a factor in certain intestinal disorders, but its frequent occurrence in the intestinal tract of healthy men and animals is not in accord with this supposition.

In animal experiments relatively large doses are required to produce lesions: less than 5 ml. of a young dextrose broth culture injected subcutaneously in guinea pigs, which are the most susceptible experimental animals, usually results in only a local manifestation. In a few hours the immediately overlying hair loosens, the skin becomes gangrenous and raised slightly over an area of subcutaneous tissue digestion in which a small amount of gas appears. Such animals usually show no systemic involvement and the lesion heals in a few days, leaving a necrotic scar which heals slowly. The reaction to intramuscular injection is only slightly more severe.

The most obviously important of the pathogenic manifestations of *Cl. sporogenes* (and probably of other putrefactive anaerobes) is that of a mutual acceleration in metabolism which occurs during growth with the more definitely pathogenic anaerobes, especially *Cl. welchii, Cl. septicum* and *Cl. novyi.* While the presence of various aerobes is in some degree stimulating to the growth of obligate anaerobes (due partly, as Pasteur suggested, to the absorption of oxygen, but also to other unknown factors) the presence of putrefactive anaerobes greatly enhances the pathogenicity of the non-putrefactive pathogens. The proteolytic forms supply protein-split products which the fermentative types are unable to elaborate so rapidly. It may be noted that, while an admixture of a proteolytic culture reduces the minimum fatal dose of a non-proteolytic anaerobic experimentally, the admixture of *sporogenes* filtrate with toxic filtrates of *Cl. welchii* or *Cl. novyi* has no such action; there may, in fact, be a diminution of toxicity.

BLACKLEG (CLOSTRIDIUM CHAUVEI)

Blackleg, also known as quarter evil and symptomatic anthrax (not to be confused with anthrax, Chapter 27), is an important, widespread, acute disease affecting cattle. It occurs wherever cattle are kept and is prevalent throughout the United States with the possible exception of the Southern Atlantic and Eastern Gulf States. The name blackleg, like gaseous gangrene, has been applied to affections due to various anaerobes; in some instances *Cl. septicum* or, rarely, *Cl. novyi* is found, but the principal cause is *Cl. chauvei.* Just as *Cl. welchii* (Type A) is not involved in natural infections of lower animals, so *Cl. chauvei* has never been shown to be responsible for any human infection.

Although the bacilli had been earlier observed and the disease transmitted by the injection of the serous fluid from an infected animal into a healthy animal, *Cl. chauvei* was cultivated and its causal relation to blackleg established by Arloing, Cornevin and Thomas in 1887.

Morphology. *Cl. chauvei* is a gram-positive, motile, sporulating rod. The size is variable, ranging from 3 to 8 μ, in length and about 1 μ in breadth. The cells occur singly as a rule; in contrast with *Cl septicum,* there is little

tendency to form chains or filaments. The spores are subterminal and oval, swelling the vegetative cell in which they occur. Sporulation is often preceded by a marked swelling of the vegetative cell. Deep agar colonies are minute, compact and downy. On the surface of blood agar well separated colonies are flat, round or leaf-like, and hemolytic.

Physiology. This bacillus is a strict anaerobe and, like *Cl. sporogenes,* grows at temperatures as high as 50° C., though the optimum is 37° C. It will grow on the usual laboratory media but is best cultivated in meat or brain medium. These are never discolored nor digested by pure cultures, but they may be slightly softened. Gelatin is liquefied, but coagulated serum and egg are not. Hydrogen sulfide is produced, but indol is not formed and nitrates are not reduced to nitrites. Dextrose, levulose, galactose, maltose, sucrose and
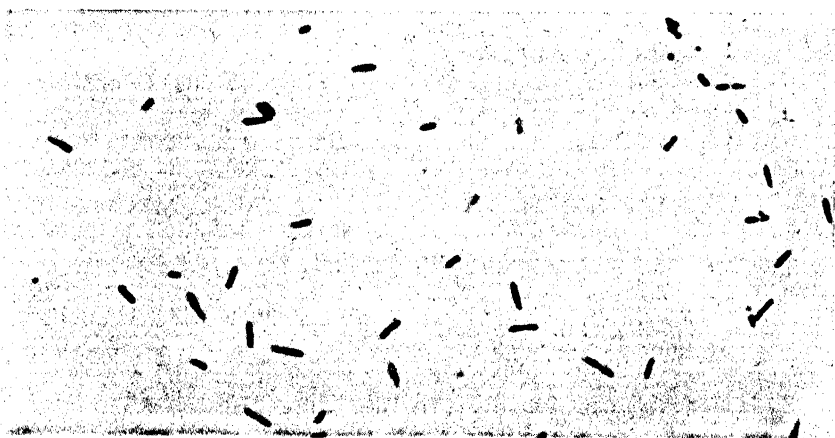
Fig. 134. *Clostridium chauvei* from pure culture. Subterminal oval spores are apparent: note the swollen pre-sporulating cell on the far right. Fuchsin; × 1050.

lactose are fermented with the formation of acid and gas; inulin, salicin, mannitol, dulcitol and glycerol are not fermented. It may be noted again that this microorganism may be differentiated from the closely related *Cl. septicum* on the basis of the sucrose and salicin fermentation. Litmus-milk cultures become acid and the casein is precipitated, but peptonization does not occur.

Pathogenicity. Natural infections due to *Cl. chauvei* occur principally in cattle. There are still several obscure features in the epidemiology. The disease occurs at special seasons of the year, is connected with certain localities, and is said to show a distinct predilection for the best young stock. The portal of entry is uncertain; whether it enters by way of minute abrasions on the skin or through the gastro-intestinal mucosa is quite unknown. Experimentally, *Cl. chauvei* is pathogenic for cattle, sheep, goats, guinea pigs and mice; horses, asses, hogs, rabbits, rats and pigeons are somewhat refractory.

The symptoms in animals consist in crepitant localized swellings, which in natural infections occur on the thighs, neck or shoulders. The animals become stupid, feverish and anorectic. Treatment is rarely successful, and sick animals usually die in one to two days. The mechanism of the disease is that of progressive bacteremia. A weak exotoxin is produced.

Immunization. A method of prophylactic inoculation which was devised by Arloing, Cornevin and Thomas has been widely used in active immunization. The Lyons[54] vaccine, as distributed by the Bureau of Animal Industry of the United States Department of Agriculture, is prepared as follows: The muscle tissue from a fresh blackleg tumor is pulverized in a mortar, extracted with water, and the extract dried at 35° C. The dry brown scale which results is suspended in water (2 parts), heated for six hours at 95° to 99° C., and injected in appropriate quantities as determined by test and specified on each package distributed. The dried material retains a high degree of activity for several years. Some commercial firms distribute this vaccine in the form of strings to be sewn into the flesh; other dispense the powdered vaccine in the form of pellets which are inoculated by means of a "pill gun." In 1901–2 (July 1 to June 30) 565,628 cattle were vaccinated in the United States. During the previous season 14,817 deaths had occurred; in a similar period after vaccination the number of deaths was only 2902.

Bacteria-free filtrates of edema fluid expressed from the flesh of guinea pigs dead of blackleg or of liquid cultures have been used as immunizing agents with promising results.[55]

Agglutinating antisera may be prepared against *Cl. chauvei*, and this species appears to be immunologically homogeneous.

CLOSTRIDIUM BOTULINUM

The type of food poisoning termed botulism has been discussed elsewhere (p. 272) and need not be considered further here. In Germany botulism was first definitely observed in 1785 and was and is associated, though by no means exclusively, with the consumption of sausages; hence the not altogether appropriate name botulism (Lat., *botulus*, sausage). The causative bacterium was isolated by van Ermengem in 1896 and named *Bacillus botulinus*. It is now known as *Clostridium botulinum*.

Morphology. *Cl. botulinum* is a large, pleomorphic, gram-positive, motile, sporulating rod, 4 to 6 μ in length and 0.9 to 1.2 μ in breadth. The cells occur singly, in pairs and in chains. There are 4 to 8 peritrichal flagella. The spores are subterminal and oval and distend the vegetative cells containing them. Spore formation is variable from strain to strain, some strains producing spores abundantly, others sparsely; but in general spore formation is best in sugar-free media.

Deep agar colonies are translucent, globular and diffuse, or flat and heart-shaped or disc-shaped, according to the consistency of the medium. Surface colonies are relatively large, 5 to 10 mm. in diameter, glistening, translucent at the edges with a thicker brownish center, filamentous, and hemolytic on blood agar.

Physiology. *Cl. botulinum* may be grown on the usual laboratory media under strict anaerobic conditions; cultivation on synthetic solutions has indicated that the amino acids cystine, leucine, lysine, glycine and proline are required.[56] Amino acids are decomposed by coupled oxidation-reduction reac-

[54] Referring to Lyons, France.
[55] *Cf.* Goss: Kans. Agr. Exp. Station Circular, 1919, 75, p. 4. The preparation of culture filtrates is considered in detail by Kelser: Jour. Agr. Res., 1918, *14*:253.
[56] Burrows: Jour. Inf. Dis., 1933, *52*:126.

tions rather than by direct oxidation.[57] Brain, meat and coagulated protein media are blackened and digested, gelatin is liquefied. Milk is peptonized. Hydrogen sulfide is produced, but nitrates are not reduced to nitrites and indol is not formed. Dextrose, levulose and maltose are fermented; the fermentation of other sugars is variable from strain to strain and type to type. The spores are highly resistant and withstand boiling for thirty minutes to twenty-two hours, and autoclaving at 120° for as long as twenty minutes.

Irrespective of the presence of fermentable sugar, a potent soluble toxin is produced which resembles other soluble toxins in most respects. It is, however, unusually stable to heat; heating to 80° C. for thirty minutes or boiling for ten minutes is required to destroy it. Botulinum toxin is unique in that it is not destroyed by the digestive enzymes of the gastro-intestinal tract and hence is
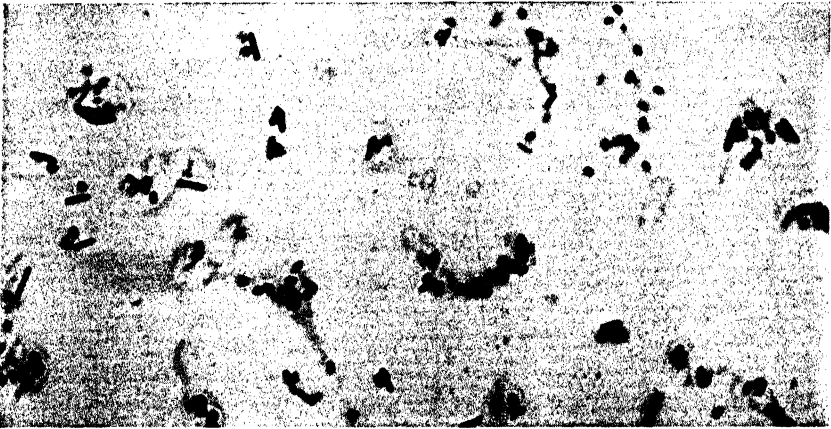
Fig. 135. *Clostridium botulinum* Type A from pure culture. Note the subterminal swollen spores and free unstained spores admixed with the vegetative cells. Fuchsin; × 1050.

effective when given by mouth. It is the most potent bacterial toxin known; the guinea-pig MLD may be as small as 1×10^{-6} ml. of broth culture.

Types. *Cl. botulinum* is subdivided into a number of types which differ from one another in that their toxins are immunologically different. The best known of these are Types A and B, which, in the past, have been regarded as solely responsible for human botulism. It is supposed that van Ermengem's original culture, now no longer available, was Type B.

Additional types have been described since the early 1920's. A toxin-producing anaerobic bacillus isolated from fly larvae (the ingestion of which was associated with a paralytic disease of chickens) has been designated Type C.[58] A closely related bacillus was isolated by Seddon[59] from botulism of cattle in Australia which he designated *Bacillus parabotulinus*. This bacillus is now designated *Cl. botulinum* Type Cβ and the fly larvae bacillus *Cl. botulinum* Type Cα. The toxins of these subtypes of Type C are related in that Cα anti-

[57] Clifton: Jour. Bact., 1940, 39:485.
[58] Bengtson: Pub. Health Repts., 1923, 38:340.
[59] Seddon: Jour. Comp. Path. and Therap., 1922, 35:147, 275.

toxin protects against both Cα and Cβ toxin, but Cβ antitoxin protects against Cβ toxin but not Cα toxin.

A South African strain first described by Theiler and Robinson,[60] designated by them *Clostridium parabotulinum equi*, has been further studied by Meyer and Gunnison[61] and given the name *Clostridium botulinum* Type D, for its toxin is not neutralized by the antitoxins of Types A, B or C. Still another type, *Clostridium botulinum* Type E, has been isolated[62] in Russia from fish, consumption of which resulted in human botulism. The Type E toxin is not neutralized by antitoxins of the other four types. There appear to be some strain differences in the action of Type E toxins though complete cross neutralization occurs.[63] Type E, it may be noted, has been found as a cause of human botulism in the United States.[64]

Toxins. As just indicated, a number of immunologically different toxins are produced by *Cl. botulinum* and a given toxin is produced by a single type rather than the various toxins being distributed among the types as in the case of *Cl. welchii*. Furthermore, the pharmacological action of the toxins is essentially the same and differentiation can be made only on an immunological basis, *i.e.*, the passive protection test in which a series of experimental animals is passively immunized, each with a single antitoxin, and all challenged with the unknown toxin.

Of these toxins, that of Type A is the most potent, exceeds in toxicity the other soluble toxins such as those of the diphtheria and tetanus bacilli, and is the most potent toxic substance known. The botulinum toxins, though protein in nature, are relatively resistant to proteolytic digestion and are thus effective when given by mouth. The Type A toxin has been prepared in crystalline form by Lamanna, McElroy and Eklund[65] and by Abrams, Kegeles and Hottle,[66] by alcoholic precipitation in the cold in the first instance, and by sodium sulfate and acid precipitation in the second. The preparations are apparently pure protein with the properties of a globulin and a molecular weight of 9 to 11 x 10^5. The LD_{50} dose for the mouse contains 4.5 x 10^{-9} mg. of nitrogen and consists of about 2.1 x 10^7 molecules. Type B toxin has also been prepared by Lamanna and Glassman[67] as a pure homogeneous protein but has not been crystallized. It is somewhat less potent than Type A toxin and the mouse LD_{50} dose contains 5 to 9 x 10^{-9} mg. nitrogen. Both pure and crude botulinum toxins may be detoxified with formaldehyde to give formol toxoid which may be used for active immunization.

The types of *Cl. botulinum* are immunologically heterogeneous, as indicated by agglutination tests, and are not biochemically or culturally distinguishable. As a group, however, they may be divided into two biochemical types, the one

[60] Theiler and Robinson: Rev. Gen. de Med. Vet., 1927, 36:193.
[61] Meyer and Gunnison: Jour. Inf. Dis., 1929, 45:106.
[62] *Cf.* Gunnison, Cummings and Meyer: Proc. Soc. Exp. Biol. Med., 1936, 35:278; Kushnir, Brun and Paikina: Ztschr. Microbiol. Epidemiol. Immunitätsf. (U.S.S.R.), 1937, 19:80 (in English 85).
[63] Hazen: Proc. Soc. Exp. Biol. Med., 1942, 20:112.
[64] Hazen: Science, 1938, 87:413; Geiger: Jour. Amer. Med. Assn., 1941, 117:22.
[65] Lamanna, McElroy and Eklund: Science, 1946, 103:613.
[66] Abrams, Kegeles and Hottle: Jour. Biol. Chem., 1946, 164:63.
[67] Lamanna and Glassman: Jour. Bact., 1947, 54:575.

proteolytic (sometimes designated ovolytic but digesting proteins other than coagulated egg albumin), whose cultural characteristics have been described above, and the other saccharolytic or fermentative in character, whose members do not hydrolyze coagulated native proteins. The proteolytic group includes Type A and some strains of Type B (the majority of the American Type B strains are proteolytic, while a great many of the European Type B strains are not). The non-proteolytic group includes some strains of Type B, and, so far as is known, all strains of Types C, D and E. Bengtson[68] has suggested that only the non-proteolytic varieties be designated *Cl. botulinum* and that the proteolytic varieties be termed *Cl. parabotulinum* (because van Ermengem's original strain was nonproteolytic)—a suggestion that has been adopted in the Bergey classification. It would appear undesirable, however, to designate practically all of the bacilli causing human botulism in the United States as "parabotulinum."

TYPES OF CLOSTRIDIUM BOTULINUM

Type	Synonym	Biochemical Character	Disease	Antitoxin
A		Proteolytic	Botulism of man, limberneck of chickens	Specific
B		Some strains proteolytic	Botulism of man, limberneck of chickens	Specific
Cα	Fly larvae bacillus	Non-proteolytic	Paralytic disease of chickens, botulism of wild ducks	Neutralizes Cβ toxin
Cβ	*Cl. parabotulinum*	Non-proteolytic	Forage poisoning of cattle (Australia)	Specific
D	*Cl. parabotulinum equi*	Non-proteolytic	Lamziekte of cattle (Africa)	Specific
E		Non-proteolytic	Botulism of man	Specific

Pathogenicity for Man. Human botulism is invariably the result of eating preserved foods in which the bacillus has grown and produced toxin. In Europe most cases have been due to the consumption of various kinds of preserved meats, such as sausage, ham, potted goose or duck and the like, while in the United States the incriminated foods have been canned vegetables for the most part. There are surprisingly few cases of botulism in this country in view of the ubiquitous distribution of *Cl. botulinum* spores in the soil—Type A has been found most commonly in the Rocky Mountain and Pacific Coast states, while Type B predominates in the Mississippi Valley, Great Lakes region and Atlantic Coast states. Type A predominates in English soils, though Type B may be found also. From 1899 to 1935 there were 261 reported cases of

[68] Bengtson: U. S. Pub. Health Ser. Hyg. Lab. Bull. No. 136, 1924.

botulism in the United States, the greatest numbers in any one year being 23 in 1922 and 22 in 1935. Over that period of time 101 cases were reported from California, by far the greatest number in any single state.

As in the case of the other sporulating anaerobes, the disease produced by *Cl. botulinum* is an intoxication; in botulism, in fact, there is no invasion of the tissues and the toxin is preformed outside the body. Under experimental conditions in which massive doses of spores have been injected, it is probable that no infection has been set up. *Cl. botulinum* has, however, been found in three cases of contaminated wounds in mixed cultures with aerobic and anaerobic bacteria, but no symptoms of botulism were apparent.[69] Under rare circumstances, then, it may proliferate in the tissues.

Until the recent reports of the occurrence of Type E, Types A and B were the only types of *Cl. botulinum* involved in human botulism. The pharmacological activities of these toxins are substantially identical; the effect appears, in the main, to be exerted on the peripheral nerve endings, possibly those of the autonomic system, and paralysis of the motor nerve end plates in the striated muscles and the diaphragm results. Whether there are pathological changes in the nerve cells of the central nervous system, such as degeneration of the Nissl bodies, is uncertain. The symptoms include vomiting, constipation, ocular paresis and pharyngeal paralysis. Death may occur within a day of the onset of symptoms or may be delayed for as long as a week. At autopsy the liver, kidneys and meninges are congested and there may be thrombosis. The case fatality is variable; in the United States it has been 60 to 70 per cent, but in Germany it is much lower, perhaps 25 per cent.

Pathogenicity for Lower Animals. Associated with human cases of botulism there have been numerous outbreaks of limberneck, a paralytic disease, among fowls fed the toxin-containing food. Other forms of botulism in lower animals occur under natural conditions, however. *Cl. botulinum* Types C and D appear to be associated exclusively with the disease in lower animals. Certain forms of forage poisoning in cattle and horses in Australia are botulism, but whether the bacilli grow and form toxin in the fodder or whether the disease results from the ingestion of rabbit carrion is not entirely clear. The South African disease of cattle, lamziekte, is botulism resulting from the ingestion of contaminated carrion. In the United Station botulism of wild ducks and other waterfowl due to Type C$_\alpha$ is prevalent and causes the death of thousands of ducks each year.[70] The source of the toxin ingested by these fowl is uncertain.

Experimentally rabbits, guinea pigs, mice, monkeys, cats and dogs are susceptible to toxin administered parenterally or *per os*. The symptoms are similar to those of naturally infected animals and of man, and the postmortem findings are much the same. Experimental animals vary widely in their susceptibility to the toxins of the various types of *Cl. botulinum*.

Immunity. Formol toxoid may be used as an immunizing antigen to produce an active immunity with circulating antitoxin present in the blood. Such active immunization has been carried out in lower animals when eco-

[69] Hall: Jour. Bact., 1945, 50:213.

[70] *Cf.* Gunnison and Coleman: Jour. Inf. Dis., 1932, 51:542. See also Kalmbach: U. S. Dept. Agr., Bur. Biol. Surv. Wildlife Res. and Manag. Leafl. BS-120. 1938.

nomically feasible; in Australia botulism of sheep and cattle has assumed sufficient proportions to justify such active immunization, and it has been applied on a small scale.[71] Man may also be immunized with fluid or alum-precipitated toxoid of Type A or Type B or a mixture of both types. Toxoid may be given in four doses at two week intervals or three doses at three to four week intervals; an arbitrarily defined protection level of 0.02 units of anti-toxin per ml. of circulating blood is reached in 50 per cent of those inoculated in about three months after initiation of the immunization.[72] Under ordinary circumstances naturally occurring botulism in man is so rare that active immunization is not worth while.

Botulinum toxin is an excellent antigen and high-titer antitoxic sera may be produced. Under experimental conditions these antitoxins have marked prophylactic value, but their therapeutic efficacy is slight. It may be pointed out that in botulism, as in tetanus, the symptoms are a consequence of the injury to the nerve tissue and the administration of antitoxin serves only to neutralize circulating toxin. The almost complete lack of therapeutic effect of botulinum antitoxin in human botulism is undoubtedly attributable to the inevitable too-late administration.

DIFFERENTIATION OF THE SPORULATING ANAEROBES

In many respects the isolation and identification of the sporulating anaerobic bacilli is somewhat more difficult than in the case of the aerobic and facultatively anaerobic bacteria. Primary cultures may be inoculated into deep brain medium and, after incubation, examined for spores, heated to 80° C. for ten minutes, then subcultured. Representative colonies may be picked from shake cultures or from the surface of plates incubated either in an anaerobic jar or in the Spray dish. A procedure for the rapid identification of the anaerobes associated with gaseous gangrene has been given in some detail by Reed and Orr.[73]

Spray[74] has divided these bacilli into main groups on the basis of reaction in iron milk[75] and has developed a key for their further differentiation and identification on the basis of morphology, physiology and pathogenicity. Of no small practical utility, his key is outlined below in an abridged form; the original paper should be consulted for details concerning other anaerobic species and the preparation of the media.

SPRAY'S KEY TO THE SPORULATING ANAEROBES

I. Iron milk: active gaseous fermentation, early coagulation (12–48 hours), no digestion of clot, no blackening.
   Lead acetate: strongly blackened.
   Nitrite +, indol −, gelatin +, motility −.
   Glucose +, lactose +, sucrose +, salicin −.

---

[71] *Cf.* Bennetts and Hall: Australian Vet. Jour., 1938, *14*:105.

[72] Nigg, *et al.*: Jour. Immunol., 1947, *55*:245; Reames, Kadull, Housewright and Wilson: *ibid.*, 1947, *55*:309.

[73] Reed and Orr: Proc. Soc. Exp. Biol. Med., 1941, 48:535; War Med., 1941, *1*:493.

[74] Spray: Jour. Bact., 1936, 32:135.

[75] Fresh whole milk is sterilized in deep tubes, each of which contains a 50 × 7 mm. piece of No. 26 gauge black stove-pipe iron.

Spores ovoid, central-excentric, not swelling rod, infrequently observed and not in fermentable sugars.

Clostridium welchii

II. Iron milk: inactive gaseous fermentation, late coagulation at 4–6 days, no digestion of clot, no blackening.
Lead acetate: no blackening, no browning.
Nitrite +, indol −, gelatin +, motility +.
Glucose +, lactose +, sucrose −, salicin +.
Spores ovoid, abundant, excentric-subterminal, swelling rod.

Clostridium septicum

III. Iron milk: inactive gaseous fermentation (long continued), late, if any, coagulation (10–20–30 days or not even at 60 days), no digestion, no blackening.
A. Lead acetate: strongly blackened.
1. Lactose −.
Nitrite −, indol −, gelatin +, motility +.
Glucose +, sucrose −, salicin −.
Spores ovoid, not abundant, excentric-subterminal, swelling rod.

Clostridium novyi

2. Lactose +.
Nitrite +, indol −, gelatin +, motility +.
Glucose +, sucrose +, salicin −.
Spores ovoid, abundant, excentric-subterminal, swelling rod.

Clostridium chauvei

B. Lead acetate: no blackening, no browning.
Nitrite −, indol −, gelatin +, motility +.
Glucose +, lactose −, sucrose −, salicin −.
Spores ovoid, not abundant, terminal, slightly swelling rod.

Clostridium botulinum C

IV. Iron milk: inactive gaseous fermentation, more or less rapid digestion (with or without previous clotting), strongly blackened early (48 hours) or late (8–9 days).
A. Lead acetate: strongly and rapidly blackened (24–48 hours).
1. Salicin +.
Nitrite −, indol −, gelatin +, motility +.
Glucose +, lactose −, sucrose −.
Spores ovoid, not usually abundant, excentric-subterminal, swelling rod. (Iron milk softly coagulated (2–5 days), first blackened (5–7 days), clot slowly digested (10–20 days).)
Differentiated by antitoxin.

Clostridium botulinum A and B

2. Salicin −.
a. Indol −.
Nitrite −, gelatin +, motility +.
Glucose +, lactose −, sucrose −.
Spores ovoid, abundant, excentric-subterminal, swelling rod. (Iron milk not coagulated, translucent then flocculent precipitate, rapidly blackened (24–48 hours), rapidly digested (8–10 days).)
(1) Tyrosine crystals not observed.

Clostridium sporogenes

(2) Tyrosine crystals in old cultures.

Clostridium tyrosinogenes

b. Indol +.
Nitrite −, gelatin +, motility +.
Glucose +, lactose −, sucrose −, salicin ±.

Spores ovoid, abundant, central-excentric, not markedly if at all swelling rod.
  (1) Pathogenic

                                               Clostridium sordellii

  (2) Non-pathogenic.

                                  Clostridium bifermentens
                              (Clostridium centrosporogenes)

B. Lead acetate: no blackening, no browning.
    Nitrite —, indol —, gelatin +, motility +.
    (Wine-red color in iron gelatin (24–48 hours).)
    Glucose —, lactose —, sucrose —, salicin —.
    Microaerophilic.
    Spores ovoid, abundant, excentric-subterminal, swelling rod.

                                    Clostridium histolyticum

V. Iron milk: no gaseous fermentation, no digestion, no blackening, coagulation late if any (15–20 days or more).
    Lead acetate: not blackened, but showing smoky browning at 24–48 hours, not measurably increased on incubation.
    Nitrite —, indol ±, gelatin +, motility +.
    Glucose —, lactose —, sucrose —, salicin —.
    Spores spherical, not abundant, terminal, swelling rod.
    A. Toxic.

                                    Clostridium tetani

    B. Non-toxic,

                              Clostridium putrificum

THE GLANDERS BACILLUS (MALLEOMYCES MALLEI)

Glanders is a disease seen, as a rule, only in the solipeds (horse, mule, ass), but is occasionally transmitted to other domestic animals, to wild animals and to man. Early regarded by many as a spontaneous, non-infectious affection, the transmissibility of glanders was demonstrated in 1837 by Rayer, who infected a horse by inoculating it with material from a case of glanders in a human subject. The causative bacillus was discovered in 1882 by Löffler and Schütz, whose work was soon confirmed and extended by Kitt, Weichselbaum and others.

Morphology and Staining. The glanders bacillus is a small rod, straight or slightly curved, usually with rounded ends, and often of irregular contour. Rather wide variations in size are observed; the average length may be taken as 2 to 5 μ and the average breadth 0.5 to 1 μ. It is often compared to the tubercle bacillus but is usually found to be somewhat broader. In culture the bacilli tend to be shorter and more uniform in size than those observed in pus smears. In pus they are sometimes found within the leucocytes but more often occur free. There is no special arrangement in such smears, but in culture the bacilli may occur in pairs and, in older cultures, produce filaments with swollen ends in which true branching may be observed. The bacilli are non-motile, non-encapsulated, and do not form spores.

Colonies on agar are small, round, convex and amorphous in consistency. They are translucent and yellowish in color and upon aging (eight to ten days) become more opaque and the center may become light brown. The growth on potato usually exhibits a characteristic appearance; clear, amber, honey-like colonies appear which may coalesce, and frequently the potato around the growth becomes tinged a greenish yellow, not unlike the discoloration produced by *Ps. pyocyanea.* On horse-blood agar the colonies are grayish green with browning of the medium but no hemolysis.

The glanders bacillus stains with the ordinary aqueous aniline dyes, though not very readily. Best results are obtained with stains containing alkali, or a mordant such as phenol (Löffler's alkaline methylene blue, Ziehl's carbol fuchsin). The bacilli are not acid-fast and are gram-negative. Cells from young cultures take the stain fairly uniformly, but those in older cultures stain irregularly, with a tendency to bipolar staining. Granules and coccus-like bodies within the cell take the stain somewhat more readily, and the bacilli not infrequently have a beaded appearance in stained preparations. Worley and Young[1] have shown that this irregular staining is due to the presence of lipid

[1] Worley and Young: Jour. Bact., 1945, 49:97.

granules which do not stain with the usual dyes but may be demonstrated with Sudan black B or iodine-fuchsin.

Physiology. Growth occurs on ordinary nutrient media but is poor and slow on primary isolation. Forty-eight hours' incubation is generally necessary for the appearance on solid media of colonies 0.5 to 1 mm. in diameter. Growth is materially enhanced by the presence of glycerol, but glucose is without effect. A slightly acid reaction is favorable and the optimum temperature is 37° C., though growth may occur over the range from 22° to 44° C. Growth on enriched media such as Löffler's serum medium or horse-blood agar is not markedly better than on glycerol agar.[2]

The glanders bacillus is quite inactive biochemically. With the exception of glucose, there is no action on the usual carbohydrates, and even the glucose fermentation is irregular and variable from strain to strain. Coagulated serum
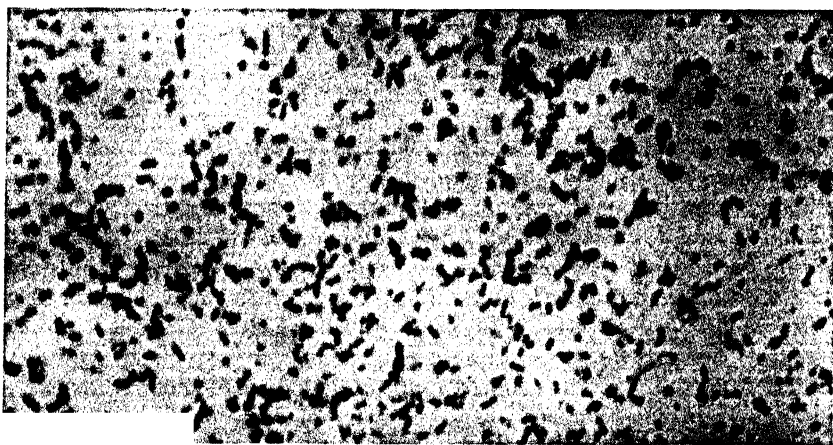
Fig. 136. *Malleomyces mallei* in pure culture. The generally poor staining is apparent and bipolar staining may be observed in some of the cells. Methylene blue; × 1250.

is not digested, but gelatin may be liquefied under appropriate circumstances by some strains at least. At temperatures at which gelatin remains solid, growth is sparse and no liquefaction is observed, but if cultures are incubated at 37° C. for twenty-six to forty days, the gelatin does not solidify on cooling. Indol is not produced, and nitrates are not reduced to nitrites; small amounts of hydrogen sulfide may be formed. Slight acid production sometimes occurs in milk, with coagulation by the tenth day of incubation and decolorization of the indicator in the lower part of the tube.

The bacillus is but slightly resistant to adverse physical and chemical agents, being readily killed by heat (55° C. for ten minutes) and bactericidal chemicals. Desiccation experiments have not given uniform results; it is said that cultures dried on threads remain viable for three or four weeks. Pure cultures appear to be more resistant to desiccation than the bacilli in the nasal secretions from diseased animals, for infected discharges are usually sterile within

[2] For recent studies on the morphology and physiology of this organism see Miller *et al.*: Jour. Bact., 1948, 55:115.

a few days. Cultures die out in four to six weeks but may be maintained by transfer on glycerol agar.

Classification. The relation of the glanders bacillus to other bacteria is by no means clear. In certain respects, such as the occurrence of branching and the favorable effect of glycerol on growth, it resembles the Mycobacteria though it is not acid-fast. It has, in fact, been grouped under the Actinomycetales by some workers in the genus Actinobacillus. In other respects it is related to the Brucella species with which it is grouped in the current Bergey classification. Generally termed *Bacillus mallei,* it was formerly designated Pfeifferella by Bergey (4th ed.) as a newly created genus. This generic name has now been discarded and a new one, Malleomyces, coined.

Pathogenicity for Lower Animals. Under natural conditions the horse is chiefly affected, but cases are occasionally observed in the carnivora (cats, dogs, menagerie animals) and in goats and sheep. Swine and pigeons are slightly susceptible. Cattle and house rats are immune. Rabbits and guinea pigs are somewhat susceptible to experimental inoculation and the hamster and ferret are the most susceptible of the usual experimental animals.[3]

Glanders manifests itself in an acute and a chronic form, which run into one another, the latter frequently terminating in an acute attack. The acute form is ushered in usually by a chill and the appearance of a high temperature in advance of any local manifestation. In a few days the mucous membrane of the nose is inflamed and becomes studded with nodules, the lymphatic system becomes largely involved, and edematous swellings appear in various parts of the body. General symptoms become more grave, and death follows in from eight to thirty days. The mule, and especially the ass, suffer commonly from the acute disease. The chronic form is the more usual type in the horse (90 per cent of cases). A great variety of symptoms and lesions have been noted in the latter animal, and the disease pursues most diverse courses in different individuals. The nasal membrane is often affected, and there is a profuse and infectious catarrhal discharge. Cutaneous glanders is known by veterinarians as *farcy,* the thickenings of the superficial lymphatics being termed "farcy buds" or "farcy pipes." In all forms of glanders there is a tendency to the production of nodules, which soften and pass over into ulcers. The glanders nodule has been considered by some writers to be structurally similar to the nodule formed by the tubercle bacillus (p. 633) but most observers are agreed that the former is a degenerative rather than a proliferative formation, and that it is radically different from the tubercle.

Experimental inoculation with pure cultures has given positive results not only in the horse, in which the characteristic features of the disease are reproduced, but in guinea pigs, field mice and other small rodents. House mice and white mice show a high but not absolute resistance, in contrast to the great susceptibility of field mice. The guinea pig responds to inoculation in a typical fashion, and has been utilized for differential diagnosis. Both in the natural and in the experimental infection the bacteria are found chiefly in the nasal secretions and in the contents of the young nodules; in the older ulcers they are relatively few in number. The blood, as a rule, contains glanders bacilli only in acute general infection.

[3] Miller *et al.:* Jour. Bact. 1948, 55:127.

Pathogenicity for Man. Veterinarians and others having to do with the care of horses are the most liable to contact glanders. Freshly isolated cultures are highly virulent, and a number of fatal infections have occurred among laboratory workers. The acute form of the malady is the more common in man, most cases terminating fatally within two or three weeks, sometimes within a few days of their inception. As in the horse, the mucous membrane of the nostrils, although not invariably affected, is a place of predilection for the glanders nodules and ulcers. Occasionally the chronic form may appear and linger for months or even years, with spreading ulceration and other features closely resembling those observed in the horse. Recovery from chronic glanders may take place, or the disease may pass into the acute stage.[4]

Path of Entrance. The avenue by which the glanders bacillus usually enters the body of the horse has not been clearly determined. The intact skin probably rarely, if ever, permits entrance, but a slight wound or injury offers a ready portal, as attested by experimentation. The mucous membrane of the nose, especially if slightly abraded, may become the portal of entry, as may the intact conjunctiva, which can be infected by contact with infectious material in two to four hours, sometimes in thirty minutes. Infection by inhalation must be rare, to judge from animal experiments, if, indeed, it ever occurs. According to Nocard, who made a special study of the mode of infection, penetration takes place by way of the alimentary tract in the great majority of cases. There is weighty experimental and other evidence in support of this view.

In man the alimentary tract is certainly not the ordinary channel of entrance; meat from glandered animals has been ingested without resulting infection. Inhalation likewise hardly enters into consideration. Probably infection through a scratch or other break in the skin is the usual origin of human cases.

Diagnosis. In prebacteriological days chronic glanders in the horse was frequently separated from other diseases only with difficulty and a considerable measure of uncertainty. At present the diagnosis of glanders is greatly facilitated by: (1) guinea pig inoculation; (2) the mallein test—(*a*) subcutaneous, (*b*) ophthalmic; (3) agglutination method; (4) the complement-fixation test.

1. *Guinea Pig Inoculation.* A male guinea pig is injected intraperitoneally with fragments of diseased tissue, scrapings from ulcers, or some of the nasal discharge from a suspected animal. A positive reaction is shown by the testicles becoming red and swollen, usually on the second or third day—*the Straus reaction.* Together with the orchitis (inflammation of the parenchyma of the testicle) there are severe general symptoms which usually culminate in twelve to fifteen days. Grayish nodules are often found in the spleen and other internal organs. The test is not absolutely specific, for Kutscher and Nocard have shown that an analogous orchitis may be produced by other organisms besides the glanders bacillus. It is often, however, of value, especially when, for one reason or another, other tests are inapplicable.

2. *The Mallein Test.* Mallein is the concentrated glycerol broth in which the glanders bacillus has grown; it is prepared in the same manner as tuber-

[4] Glanders in man is reported only occasionally; see Panja and Chatterjee: Indian Med. Gaz., 1943, 78:150; Howe and Miller: Ann. Int. Med., 1947, 26:93.

culin. The mallein reaction consists in a rise of temperature, accompanied by a pronounced local reaction, and in many cases, though not invariably, by more or less profound constitutional disturbances.

(*a*) Subcutaneous injection of mallein (the size of the dose varying according to the concentration) into a glandered horse is followed by the signs above noted, while in an animal not infected with glanders the temperature is slightly or not at all affected and the general symptoms are absent. The temperature of the suspected animal should be taken at two-hour intervals before the injection is made, and after the injection at the ninth, twelfth, fifteenth and eighteenth hours, at least. The increase of temperature in glandered horses varies from 1.5° to 2.5° C. above the normal, and is distinctly high on the second day after injection. Healthy horses often show a distinct temperature increase on the first day after inoculation, but as a rule this disappears quickly.
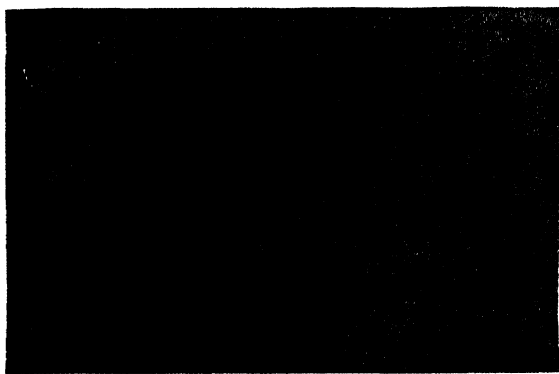
Fig. 137. Glanders bacillus in giant cells and exudate of human lungs (Coleman and Ewing).

In the use of mallein, as in the tuberculin test, care must be taken to exclude other influences that disturb the normal temperature relations. Experienced observers lay much stress upon the appearance of swelling at the seat of inoculation. In a glandered animal the tumefaction is large, hot and painful; it increases in size up to twenty-nine to thirty-six hours, persists for about a week, and gradually disappears. In a healthy animal a swelling may occur, but it is never large and vanishes within twenty-four hours.

There is complete agreement among veterinarians regarding the high diagnostic value of mallein. The reaction is specific, is usually sharp and decisive in character, and almost never fails to reveal the presence of infection. Nocard expressed himself very emphatically: "A complete mallein reaction is unequivocal; the animal that reacts is glandered. An animal which does not react to an injection of mallein is not glandered, whatever the character of the symptoms." Recent workers prefer the ophthalmic test to the subcutaneous test.

(*b*) The ophthalmic test consists in the introduction of the mallein, preferably in tablet form, into the conjunctival sac. The reaction is very reliable, and the test can be readily made by every practicing veterinarian.

3. *The agglutination method* has had extensive official use in Prussia and

21

Austria. The serum of normal horses agglutinates in dilutions of from 1 : 200 to 1 : 300, and the reaction is specific only when rather high dilutions (1 : 500 to 1 : 3200) are used. The serum from sound animals, however, sometimes agglutinates the glanders bacillus in a dilution as high as 1 : 500. Occasionally the reaction fails to appear in the serum of glandered animals. The test is liable to the usual difficulties and source of error in the hands of an unskilled observer.

4. *The complement-fixation test* is very accurate but demands special laboratory facilities and is less easy to apply in a practical way than the ophthalmic test.

Immunity and Prophylaxis. Permanent immunity to glanders can neither be conferred by an attack of the disease nor produced by an artificial means. Nocard fed with infectious matter three horses which had previously recovered from the disease, and found that these animals showed no resistance superior to that of a healthy control animal. Similarly, Lobel, Schaaf and Roza[5] were unable to produce an immunity in guinea pigs or horses by the inoculation of bile-attenuated avirulent bacilli. Chronic glanders may exist for years, and is in no wise a warranty against the sudden development of an acute attack.

No very potent nor characteristic toxic substance has been obtained from cultures of the glanders bacillus, and attempts at immunization with the products of this organism have been eminently unsuccessful. It is stated by a number of observers that repeated injections of mallein will exercise a curative action upon certain forms of recent infection, but experimentally mallein is without immunizing power. The sera of animals treated with mallein injections and the sera of naturally immune animals, such as cattle, are, according to most observers, totally devoid of any preventive or curative value. The most that has been accomplished in the way of immunization is a very moderate augmentation of resistance in dogs injected with small non-fatal doses of living cultures.

The experience of Great Britain shows that the disease may be practically eradicated by slaughtering every animal showing clinical signs of glanders or giving a positive mallein test, and properly disposing of the carcass. By this means the number of horses affected was cut down from 2012 in 1906 to 2 in 1925.

MALLEOMYCES WHITMORI (MALLEOMYCES PSEUDOMALLEI)

Melioidosis, a disease of rodents somewhat similar to glanders, is caused by *Malleomyces whitmori*, a microorganism which closely resembles the glanders bacillus. It has been observed in Rangoon, where it is thought to be primarily a disease of the wild rat and is occasionally communicable to man. *M. whitmori* differs from *M. mallei* in that it is actively motile, liquefies gelatin and attacks carbohydrates more energetically. It grows considerably more rapidly and its colonies on glycerol agar develop a wrinkled, corrugated surface and are quite different in appearance from those of *M. mallei*. A second colony type may be produced, however, which is very similar to the

⁵ Lobel, Schaaf and Roza: Nederland. Indische Blad. Diergeneesk., 1941, 53:100.

colonies of the glanders bacillus.[6] *M. whitmori* is thought by some to be closely related to *Pseudomonas pyocyanea* in many respects and strains have been reported[7] which produce pyocyanin.

The charateristic lesion of the disease is a small caseous nodule which is found in man in almost any part of the body except the brain. The nodules may coalesce to form large areas of caseation in some cases and in others break down into abscesses. There is some reason to believe that melioidosis in man is often traumatic in origin, either the bacilli entering the injured tissues or the defense mechanism being broken down in part by the injury.[8] Transmission of the infection among guinea pigs by biting insects, mosquitos and fleas, has been reported.[9] Rodents usually die within a short time from septicemia, and the lesions appear as small nodules superficially resembling tubercles. Guinea pigs and rabbits are highly susceptible to inoculation. *M. whitmori* also produces the Straus reaction.

[6] See Finlayson: South African Med. Jour., 1944, *18*:113.

[7] Blanc, Delage and Martin: Ann. Inst. Pasteur., 1943, *69*:65.

[8] *Cf.* Le Moine, Hasle and Nguyen-Duc-Khoi: Bull. Soc. Med.-Chirurg. Indochine, 1937, *15*:662; Toullec and Huard: *ibid.*, 1937, *15*:667; Sudibyo: Geneesk. Tijdschr. v. Nederl.-Indie., 1938, 78:1424 (English summary).

[9] Blanc and Baltazard: Ann. Inst. Pasteur, 1942, *68*:281.

CORYNEBACTERIUM (THE DIPHTHERIA BACILLUS)[1]

As a clinical entity diphtheria dates from the observations of Bretonneau in 1826. The diphtheria bacillus was observed and described by Klebs in 1883, but its etiological relation to the disease was suggested by the investigations of Löffler the following year. Löffler isolated the bacillus observed by Klebs in pure culture from a number of cases of diphtheria but expressly disclaimed the assumption that his bacillus was the causal agent of diphtheria, in part because he found it in the throat of a healthy child, and in part because he did not find it in all cases of what were apparently clinical diphtheria. The significance of Löffler's findings is now clear, however, for it is known that other bacteria, such as streptococci, can produce a condition in the throat closely resembling diphtheria and that the diphtheria bacillus is not infrequently present in the throat of healthy carriers. Further investigations by other workers indicated that the Klebs-Löffler bacillus was always present in the typical false membrane of diphtheria. In 1888 Roux and Yersin showed that this bacillus formed a soluble toxin which reproduced the characteristic symptoms and lesions of diphtheria and thus demonstrated its etiological relation to the disease.

Morphology and Staining. The diphtheria bacillus is a slender rod ranging from 1 to 6 μ in length and 0.3 to 0.8 μ in breadth. The bacilli are highly pleomorphic, for, in addition to the straight or slightly curved rods, club-shaped and branching forms are not infrequently observed. The presence of the latter, which are a consequence of true branching, is indicative of the close relation of the diphtheria bacillus to some of the higher fungi, and it is classified with the actinomycetes rather than under the Eubacteriales or "true bacteria." Upon completion of cell division a movement designated as snapping occurs, and the bacilli may remain attached but at sharp angles to one another.

The diphtheria bacillus exhibits a marked tendency to stain irregularly. Some cells stain solidly, others take the stain more deeply in transverse bands to give a barred appearance, and in still others deeply staining metachromatic or Babes-Ernst granules are found. A single cell may contain from one to generally not more than five or six such metachromatic granules; they may be found at one or both ends of the cells, particularly those with swollen ends, and when more than two are present the remainder are scattered within the

---

[1] Diphtheria is considered in detail by Andrewes et al.: *Diphtheria: Its Bacteriology, Pathology and Immunology*, Medical Research Council (British), London, 1923; and by Forbes: *Diphtheria, Past and Present; Its Aetiology, Distribution, Transmission and Prevention*, John Bale, Sons & Danielsson, London. 1932. See also Corson: *Diphtheria: A Summary of Recent Literature*, Bull. Hyg., 1943, 12:969.

cell substance. This irregular staining is apparent with Löffler's alkaline methylene blue or with toluidine blue; Neisser's stain[2] is regarded by many workers as producing an even greater contrast between the heavily and lightly staining portions of the cell substance. Morton and Francisco[3] have shown that the metachromatic granules are more readily differentiated when the basic dye is made up in an acid solution.

In stained smears the appearance of diphtheria bacilli is highly characteristic. They may not be identified on morphological grounds alone, however, for many of the pseudodiphtheria bacilli or diphtheroids stain in the same irregular fashion and are similarly pleomorphic. It was formerly thought that there was an association between morphological type and virulence. At present, however, little emphasis is placed upon morphology in this connection, though in a
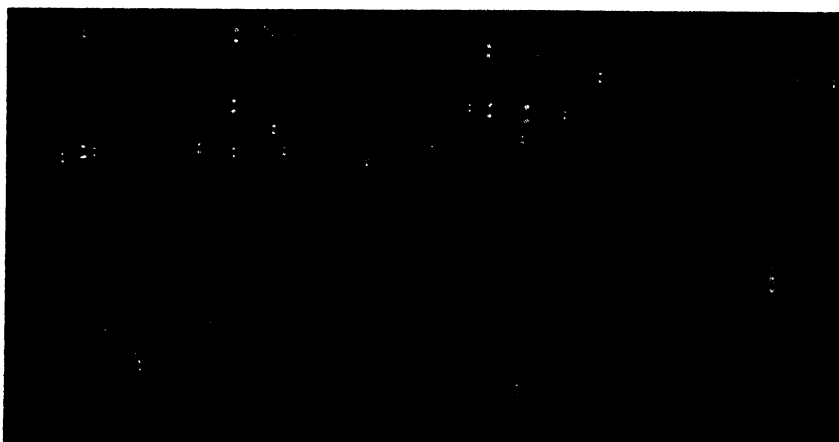
Fig. 138. Colonies of *Corynebacterium diphtheriae* on blood agar. Note the smooth, raised translucent appearance and relatively small size. × 2.

general way granular types seem to predominate in clinical diphtheria and there appears to be a rough association between morphological type and the *mitis* and *gravis* types, as will appear. The diphtheria bacillus is gram-positive but decolorizes more readily than most of the gram-positive bacteria.

Surface colonies on Löfflers' serum medium or on agar are small and gray; when viewed under low magnification they are found to be coarsely granular and somewhat irregular in outline, with ragged or fringed edges. On differential media containing potassium tellurite, colonies of the diphtheria bacillus are dark gray or black because of reduction of the tellurite and readily differentiated from those of contaminating bacteria. Tellurite reduction apparently occurs within the bacterial cell.[4] It may be noted that although the characteristic morphology of the diphtheria bacillus is apparent in stained smears from colonies on Löffler's medium, smears from colonies on tellurite media are often not characteristic.

[2] The bacilli are stained with an acid methylene blue solution followed by Bismarck brown.

[3] Morton and Francisco: Stain Technol, 1942, 17:27.

[4] Morton and Anderson: Proc. Soc. Exp. Biol. Med., 1941, 46:272.

Physiology. The optimum growth temperature for the diphtheria bacillus is 34° to 36° C. and it grows well at 37° C.; growth will take place over the range from 15° to 40° C. An alkaline reaction is required, pH 7.8 to 8.0, and free access to air is essential, for growth under anaerobic conditions is sparse.[5]

In primary isolation the diphtheria bacillus is best cultivated on enriched media. Growth is rapid on Löffler's serum medium (3 parts of beef or sheep serum and 1 part of 1 per cent dextrose broth coagulated in slant form by inspissation) and minute but visible colonies appear after twelve to twenty-four hours' incubation. In recent years a variety of differential and selective media have been introduced, all of which contain potassium tellurite. The better known of these are the chocolate agar-tellurite medium of Anderson and his co-workers,[6] which has been subject to minor modifications by a number of other workers, such as Neill's medium and Hoyle's medium which are

Fig. 139. The diphtheria bacillus, *gravis* strain, pure culture on blood agar. Methylene blue stain. Note the bipolar staining and the club-shaped forms. The lightly stained cells with deeply stained areas are characteristic of *gravis* morphology. × 1200.

used in England, and the various media developed by Clauberg, whose inspissated serum-glycerol-tellurite medium has been widely tested. There is general agreement that the proportion of positive cultures is somewhat higher with the Clauberg medium than with Löffler's medium; whether the heated blood-tellurite media are superior to Löffler's medium is not clear. As indicated above, the characteristic morphology of the diphtheria bacillus is not always seen in smears from colonies on tellurite media and, therefore, in some laboratories both Löffler's medium and a differential tellurite medium are inoculated and the former used for microscopic examination if typical colonies appear on the differential medium.

Enriched media are not, however, essential to the growth of the diphtheria bacillus, for this microorganism can be cultivated on ordinary nutrient and in-

[5] Strains of virulent diphtheria bacilli which grow more luxuriantly under anaerobic conditions than in the presence of air have, however, been reported. *Cf.* Emilio: Giorn. di Batteriol. e Immunol., 1938, 21:256.

[6] Anderson, Happold, McLeod and Thomson: Jour. Path. Bact., 1931, 34:667.

fusion media. Growth is somewhat scanty on the former but good on the fresh meat infusions. The intensive investigations of Mueller[7] have shed considerable light on the growth requirements of these bacilli. Some strains, including the well-known Park 8, may be cultivated on synthetic solutions containing a number of amino acids together with small quantities of nicotinic acid, β-alanine or pantothenic acid, and pimelic acid. It has been suggested that pimelic acid is utilized by this bacterium for the synthesis of biotin since growth is stimulated by biotin in the absence of pimelic acid. Recently isolated strains also require oleic acid for development, especially if the inoculum is small. Nutritive requirements differ somewhat from one strain to another and a general statement is not possible.

The diphtheria bacillus does not liquefy gelatin or digest coagulated protein. Indol is not formed[8] and nitrates are reduced to nitrites. All strains form acid
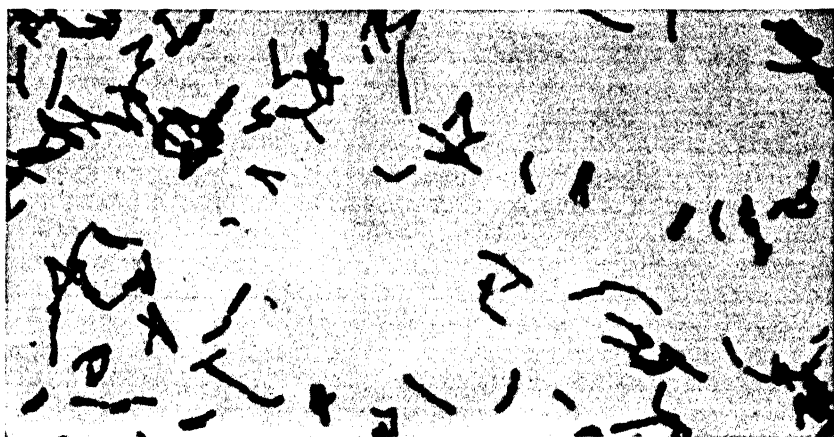
Fig. 140. The diphtheria bacillus, *intermedius* strain, pure culture on blood agar. Methylene blue stain. Note the irregular staining and barred appearance characteristic of the *intermedius* variety. $\times$ 1200.

but no gas from dextrose and levulose, and some strains ferment dextrin, glycogen, starch, galactose, maltose and glycerol. There appear to be no well defined biochemical groups among these bacilli. The fermentation of dextrose is of some interest in that propionic acid is formed. Other products of the fermentation include lactic, acetic, formic and succinic acids and ethyl alcohol.

In ordinary culture media the diphtheria bacillus may retain its vitality for relatively long periods of time. It will live six to eight weeks on agar, five to six months on blood serum, twelve to fifteen months on dextrose blood serum, and as long as three months in particles of diphtheric membrane. Although virulence is ordinarily reduced by continued culture on laboratory media, some strains remain fully virulent, *i.e.*, toxigenic, on prolonged cultivation; Löffler recorded one instance in which virulence was maintained over 77

[7] Summarized by Mueller: Jour. Bact., 1938, 36:499; Bact. Rev., 1940, 4:97.

[8] The test with sulfuric acid and potassium nitrite may be positive because of the formation of indol-acetic acid, but no color is produced with Ehrlich's reagent, *p*-dimethylamidobenzaldehyde. (Frieber: Centralbl. f. Bakt., 1921, 87:254.)

transfers covering a period of twenty-seven months. The bacilli are unusually susceptible to heat; a suspension or broth culture is killed by holding at 58° C. for ten minutes. In diphtheric membrane they are considerably more resistant.

Toxin. With the possible exception of the Shiga dysentery bacillus, the diphtheria bacillus is the only aerobic bacterium that produces a powerful exotoxin comparable to those formed by the sporulating anaerobes. Filtrates from broth cultures are not so toxic as those of the tetanus and botulinus bacilli; exceptionally potent filtrates may contain as much as 1000 guinea-pig MLD's per milliliter. It may be noted that in regard to the diphtheria bacillus virulence and toxigenicity are synonymous. *The virulence test,* the inoculation of guinea pigs with broth culture of the bacillus, is, then, a test of the ability of the bacilli to form toxin.

The production of toxin by the diphtheria bacillus is markedly influenced by environmental and nutritive conditions; even strongly toxigenic strains may produce little or no toxin under unfavorable conditions. A slightly alkaline reaction, pH 7.8 to 8.0, is essential, for an acid reaction strongly exhibits toxin formation. Free access to air is also necessary, and for the production of toxin the bacilli are cultivated in thin layers of beef infusion broth. Maximum amounts of toxin are found after seven to ten days' incubation at 36° to 37° C.

An infusion medium, beef infusion, containing adequate amounts of peptone (2 per cent) has long been regarded as essential to the production of maximum amounts of toxin. The marketed brands of peptone are variable in this respect, some being much better for the production of toxin than others. The presence of protein or large peptone molecules is not essential to toxin formation, however, and potent toxins may be produced in chemically defined media containing appropriate amino acids and other compounds. The critical factor is not the quality of peptone or other source of nitrogen, as once thought, but the concentration of iron in the medium. Pappenheimer and Johnson[9] found that maximum toxin production occurs only over a narrow range of iron concentration, the optimum being 0.14 μg./ml., and 5.0 μg./ml. almost completely inhibits its formation. Calcium, sodium and potassium also affect toxin production but the concentrations are not so critical. Mueller[10] has suggested that the small amount of toxin produced in the presence of large amounts of iron represents normal production while the increased production under conditions of iron starvation is possibly the result of a compensatory mechanism in which the toxin molecule takes part in some process ordinarily catalyzed by an iron-containing enzyme. Pappenheimer has reported[11] that four mols of porphyrin and one mol of toxin disappear from the filtrate for every four mols of iron added to the culture medium, suggesting that the toxin is the protein moiety of an iron porphyrin respiratory enzyme of the diphtheria bacillus. However this may be, potent diphtheria toxin is formed in a reproducible semi-synthetic medium containing hydrolyzed casein, nicotinic and pimelic acids, cystine, maltose, calcium and iron that gives better and more consistent toxin production than the complex infusion media.[12]

[9] Pappenheimer and Johnson: Brit. Jour. Exp. Path., 1936, 17:335.
[10] Mueller: Jour. Immunol., 1941, 42:343.
[11] Pappenheimer: Jour. Biol. Chem., 1947, 167:251.
[12] Mueller and Miller: Jour. Immunol., 1941, 40:21.

The properties of the diphtheria toxin are similar to those of the other soluble toxins which have been discussed elsewhere (p. 202) and need not be considered at length here. Suffice it to say that the toxin is unstable to slight acidities, *i.e.*, a *p*H of 6 or less, is heat-labile and apparently protein in nature. The production of toxin in synthetic nutrient solutions containing insignificant amounts of high molecular weight substances allows its separation in a relatively pure form by salting out and dialysis. A protein in a high state of purity and having a molecular weight of about 72,000 has been prepared from such cultures by Pappenheimer[13] and is regarded by him as identical with diphtheria toxin. The guinea-pig MLD of this material is about 0.001 mg.

Diphtheria toxin is an excellent antigen and gives rise to high titer antitoxic sera. The standardization of diphtheria toxin and antitoxin is discussed elsewhere (p. 288). It has been noted from time to time that various substances, including bile, ascorbic acid (vitamin C), sterols such as lanolin, cholesterol and the like, will neutralize diphtheria toxin. The significance of such observations is not entirely clear. Generally relatively large amounts are required to neutralize small quantities of toxin. One or two attempts have been made to utilize ascorbic acid in the treatment of clinical diphtheria but without significant results.

It may be noted that there is great variation from strain to strain of diphtheria bacilli in the ability to produce toxin, ranging from the highly toxigenic strains to relatively or completely atoxigenic ones. The well known Park No. 8 strain is one of the good toxin producers and has been widely used for this purpose. It has been observed by some workers that pure cultures of toxigenic strains will, at times, give rise to weaker toxin producers or completely atoxigenic varieties.

Variation. As in other groups of bacteria, smooth and rough variants of the diphtheria bacillus have been observed and the type of colony formation has been found to be correlated with morphology and virulence; the S variant is the more virulent and the form commonly found in acute cases of diphtheria. Morphological and biochemical variation observed in the diphtheria bacilli has been critically reviewed by Morton.[14] As a consequence of its pleomorphism and tendency to branching and the like, the diphtheria bacillus has been thought by some to undergo the cyclic transformations of a complex life cycle including the production of gonidial and filterable forms. This concept is not generally accepted. It has also been suggested that some of the diphtheroid bacilli are diphtheria bacilli which have lost their ability to form toxin.

Types. Morphological types of the diphtheria bacillus were described by Anderson and others[15] in England in 1931 and since have been found in various parts of the world. These were of very considerable interest when first observed for there appeared to be an association, especially in England, between the type and the degree of severity in the clinical manifestations of the disease. Those designated as the *gravis* and *intermedius* types were found in severe cases of diphtheria, and the *mitis* type in the milder cases. The associa-

---

[13] Pappenheimer: Jour. Biol. Chem., 1937, *120*:453; see also Linggood: Brit. Jour. Exp. Path., 1941, 22:255.

[14] Morton: Bact. Rev., 1940, 4:177.

[15] See the comprehensive review by McLeod: Bact. Rev., 1943, 7:1.

tion was less clear on the Continent, and there appeared to be little or no relation in the United States, where the *mitis* type occurs much more frequently and perhaps only 1 per cent of the strains are of the *gravis* type. The toxins produced by the three types are equally neutralizable by the ordinary antitoxic sera, but the *mitis* strains produce toxin somewhat more actively *in vitro* than the *gravis* and *intermedius* types. The three types also appear to be equally virulent for the guinea pig. By now it is more or less generally agreed that the differentiation of these types is not significantly related to clinical severity, but has been useful from an epidemiological point of view.

These types may be differentiated by their colonial form on tellurite media. The *gravis* type produces irregular striated colonies predominantly gray in color; the *mitis* type, small, round, smooth, convex colonies predominantly
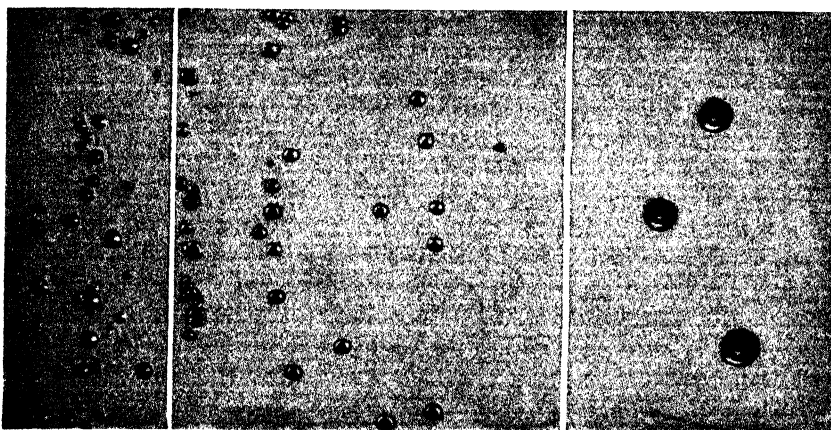
Fig. 141. The varieties of the diphtheria bacillus on chocolate-tellurite agar. Left, *mitis* type; note the characteristic raised, small black colony. Center, *intermedius* type; the lighter color, beginning radial striation and small size are apparent. Right, *gravis* type; the gray color, larger size, raised center and radial striation are evident.

black in color and softer in consistency; and the colonial form of the *intermedius* type lies between these. Colonial differences are also apparent on certain other media, such as trypsin-serum agar and a potato extract-cystine-water blue-glycerol medium devised by Clauberg. On fresh blood agar the *mitis* type is usually hemolytic, the *intermedius* type non-hemolytic, and the *gravis* type usually non-hemolytic. A further distinction is the fermentation of glycogen and starch by the *gravis* type; but other biochemical tests do not differentiate these types.

There is some association between colonial type and the morphology of the bacillary forms. Those of the *gravis* type show one or two deeply staining areas, the remainder of the cell staining very lightly; metachromatic granules are seldom observed. Bacilli of the *mitis* variety stain irregularly and contain very many well-developed metachromatic granules. The *intermedius* forms exhibit the familiar barred appearance. While some 80 per cent of the *intermedius* variety conform to this morphology, only 50 to 60 per cent of the *gravis* strains are typical, the remainder resembling the *mitis* and *inter-*

medius forms. Furthermore, 5 to 20 per cent of the *mitis* strains show barred forms.

Serological investigation has shown that while these three types are antigenically distinct from one another, the types are not necessarily homogeneous. The *mitis* strains are heterogeneous, the *gravis* strains fall for the most part into two types, and the *intermedius* strains are relatively homogeneous, some strains showing relationship to the *gravis* types.

Not all strains showing the morphological and biochemical characteristics of these types are virulent, *i.e.*, toxigenic, diphtheria bacilli. Since the diphtheria bacillus is differentiated on the basis of the formation of immunologically specific toxin, it is apparent that *mitis, gravis* and *intermedius* types of diphtheroid bacilli occur. In the series studied by Frobisher,[16] for example, only 10 per cent of the strains typed as *gravis* were toxigenic. Furthermore, a certain proportion of toxigenic strains cannot be allocated into one or another

CHARACTERISTICS OF THE DIPHTHERIA BACILLUS TYPES

Type			*mitis*	*intermedius*	*gravis*
Morphology	Microscopic		Usually long, with many metachromatic granules—80 per cent typical	Usually barred, club forms common—80 per cent typical	Short, evenly staining —50-60 per cent typical
	Colonial	Tellurite	Small, round, smooth, convex, black with grayish periphery	Small, flat, dull, gray, raised center	Large, irregular, dull, gray, raised center, radial striations
		Chocolate	Smooth, semi-opaque, glistening	Flat, dry, opaque, slight greenish zone	Flat, dry, matt, opaque
		Broth	Uniform turbidity, sometimes slightly granular, soft pellicle	Finely granular turbidity	Granular, flakes, pellicle—variable
Physiology	Fermentation of	Glycogen	—	—	+
		Starch	—	—	+
	Hemolysis		+	—	±
Immunology			Heterogeneous	Relatively homogeneous	Two main types

of these types. The proportion of indeterminate strains is said to be higher when diphtheria is mild. Anderson *et al.*[6] found about 5 per cent of their strains (British) were not typable and 44 per cent of the strains found by Seligmann[17] in New York City in 1940 were indeterminate.

Pathogenicity for Man. Diphtheria is primarily a disease of childhood and the age incidence is an expression of waning passive immunity of maternal origin and the development of an active immunity on the one hand, and the risk of exposure on the other. The very young child is passively protected and not exposed to great risk of infection, but by school age the immunity has disappeared in large part and risk of exposure to infection is tremendously increased with entrance into school. The adolescent and adult have acquired an active immunity as a consequence of clinical or, more commonly, inapparent

[16] Frobisher: Amer. Jour. Pub. Health., 1942, 32:709.
[17] Seligmann: Amer. Jour. Hyg., Sec. B, 1941, 34:125.

infection. Thus clinical diphtheria is most common in the five to fourteen age group though the carrier state and inapparent infection are probably no more so than in the higher age groups. Though diphtheria is in large part preventable by artificial immunization, particularly of the preschool child, it continues to be an important disease and 17,612 cases with 1494 deaths were reported in the United States in 1945.

Diphtheria in man is usually a local infection of the mucous surfaces. The pharynx is most commonly affected, but infection of the larynx, or membranous croup, and nasal diphtheria, or membranous rhinitis, are not infrequently observed. Diphtheritic infections of the conjunctiva and of the middle ear are less common and cutaneous or wound diphtheria is only occasionally observed. The last, however, may assume considerable proportions under certain circumstances. Ulcerative diphtheria of the skin, sometimes called desert sore or tropical ulcer, has been observed in epidemic form in Haifa,[18] and ulcers of the deep, punched out type occurred with some frequency in troops living under combat conditions in the south and central Pacific areas during World War II.[19] Infection of the mucous surfaces of the genital organs is occasionally found. The invasion of other localities is rare; primary infection of the lungs and diphtheritic meningitis have been observed, and infection of the umbilicus in the new-born has been reported.

It has long been known that diphtheria bacilli are not often found in the internal organs, but the frequency with which such infection occurs is not definitely known. Diphtheria bacillus septicemia is occasionally observed,[20] however, and it is of interest to note here that a few cases of acute vegetative endocarditis caused by the diphtheria bacillus have been reported.[21]

The symptoms and lesions produced are due partly to the presence of the bacillus and partly to its toxin. The chief local consequence of infection is a degeneration of the epithelial cells, extending to the underlying tissues and accompanied by a profuse fibrinous exudation, and the characteristic diphtheritic membrane, containing fibrin, dead tissue cells, leucocytes and bacteria, is formed on the affected surface. The mechanical interference of the membrane with breathing may assume significant proportions and even necessitate intubation or tracheotomy.

Although diphtheria toxin undoubtedly plays a part in the formation of the membrane, its systemic effects following absorption are by far the most important, and diphtheria is, like tetanus, essentially a toxemia. The organs most severely affected are the kidneys, heart and nerves. A variety of lesions may be found in the kidneys, an acute interstitial nephritis being the most common. The lesions in the heart consist commonly of a fatty degeneration in the muscle fibers, which may be very extensive. Fatty degeneration also occurs both in the myelin sheath of the peripheral nerves and in the white matter of the brain and cord. These changes in muscle and nerve account for the serious cardiac weakness often observed in diphtheria and the frequent occurrence of the more or less extensive paralysis which so commonly follows an attack

[18] Gill: Arch. Dermat. Syph., 1945, 51:243.
[19] Liebow, MacLean, Bumstead and Welt: Arch. Int. Med., 1946, 78:255.
[20] The literature is reviewed by Kaschel: Ztschr. f. Kinderheilk., 1938, 59:437.
[21] Cf. Buddingh and Anderson: Arch. Int. Med., 1937, 59:597.

of the disease. It is probable that a small amount of toxin can cause extensive damage in these tissues.

Pathogenicity for Lower Animals. Diphtheria is not a natural disease of lower animals. There is a popular belief that cats may become infected and disseminate the bacilli, but this is not true. Both the local and general symptoms of human diphtheria in man can, however, be reproduced by animal inoculation. Inoculations upon the healthy mucous membrane of most adult animals lead to no changes, but if young animals be injected intratracheally, or if the mucous surface be injured before inoculation, a characteristic false membrane is produced which is histologically identical with that found in man.

The subcutaneous inoculation of a guinea pig with a sufficient amount of a young broth culture or toxic filtrate will produce death in one to four days, the time depending upon the size of the inoculum. The animal becomes obviously ill twelve to eighteen hours after inoculation, and nephritic symptoms, paralytic manifestations and other characteristics of human diphtheria are often observed. Postmortem findings include an edema and possibly necrosis at the site of inoculation, congestion of the regional lymphatics and abdominal viscera, a pleural exudate and, characteristic of diphtheritic toxemia in this animal, an enlarged and hemorrhagic condition of the adrenals. As a rule, the bacilli remain localized and are not found in large numbers in the internal organs of the infected animal. Guinea pigs that receive smaller doses and do not die by the fourth day may develop paralytic symptoms and cachexia and die later on, a condition obviously different from the acute toxemia.

Animals vary considerably in their susceptibility to infection. Rats and mice are relatively refractory; rabbits are less susceptible than guinea pigs; cats, dogs and pigeons are highly susceptible. Paralytic manifestations appear more frequently in dogs and pigeons than in guinea pigs or rabbits. Frobisher[22] has found that young chicks are susceptible to virulent diphtheria bacilli and their toxin and may be used instead of guinea pigs for the virulence test.

Bacteriological Diagnosis of Diphtheria. To establish a diagnosis of infection with diphtheria bacilli, in either case or carrier, the bacillus must be isolated and its toxigenicity demonstrated. The specimen is taken on a swab, either plain or previously dipped in sterile horse serum which is coagulated on the surface by twirling in a flame. It is best to inoculate two media, Löffler's serum agar and a tellurite medium such as chocolate tellurite agar; if only a single medium can be used, tellurite is preferable. A blood agar plate should be inoculated as well, both for the isolation of diphtheria-like colonies and to provide for the cultivation of hemolytic streptococci which may be present. After the plates have been inoculated a smear may be made by rolling the swab on a slide, and stained with alkaline methylene blue; it will serve to show the presence of the spirochetes and fusiform bacilli of Vincent's angina should these be present.

Diphtheria bacilli grow up in eighteen to twenty-four hours' incubation. If the characteristic black or grey colonies appear on tellurite, smears may be made from such colonies and from the Löffler slant for microscopic examination; the morphology of the diphtheria bacillus is frequently not characteristic on

[22] *Cf.* Frobisher, Parsons and T'ung: Amer. Jour. Hyg., 1942, 35:381; T'ung: *ibid.*, 1945, 41:57.

tellurite as indicated above. It is often inferred that only diphtheria bacilli grow as black colonies on tellurite medium. This is not true, for any bacterium that reduces tellurite will produce similar colonies; tellurite-reducing bacteria, other than diphtheria bacilli, from the nose and throat are usually staphylococci or micrococci and as a rule their colonies resemble those of the *mitis* variety of diphtheria bacillus but are blacker.

If morphologically typical bacilli are found toxigenicity must be tested by animal inoculation. This is ordinarily carried out in the guinea pig by sub-cutaneous or intracutaneous inoculation. In the first instance the growth from a Löffler slant is suspended in 10 ml. saline and 4 ml. injected subcutaneously into each of two guinea pigs, one of which has received 250 units of diphtheria antitoxin twenty-four hours previously. The diphtheria bacillus will kill the unprotected pig in three to five days and autopsy will show local edema and the characteristic hemorrhagic enlarged adrenals, while the protected animal will survive. For the intracutaneous test the growth from a Löffler slant is suspended in 20 ml. saline and 0.15 ml. injected into the shaven abdominal skin of each of two pigs as above. Toxigenicity is indicated by the development of a local infiltrated lesion which shows superficial necrosis in two or three days in the unprotected pig. By the latter technique a number of tests may be carried out in the same pair of animals.

The virulence test may also be carried out in the rabbit. The growth from a Löffler slant culture is suspended in 2 to 3 ml. of sterile infusion broth, and 0.1 ml. injected intradermally. Four hours later the animal is given 1000 units of antitoxin intravenously, and immediately afterwards a second intradermal inoc-ulation of 0.1 ml. of the bacterial suspension is made at a site adjacent to that of the first inoculation. Reactions should be read at 72 hours. If the strain of bacteria is toxigenic, the site of the first inoculation will be a central necrotic area, usually hemorrhagic, surrounded by a zone of erythema. The inoculation of antitoxin does not affect a reaction to the first inoculation, but does spe-cifically inhibit a reaction to the second inoculation, and the site of the latter appears as a small, pinkish papule. Eight to ten such virulence tests may be car-ried out simultaneously in the same animal.

Immunity. Immunity to diphtheria, arising as a consequence either of recovery from a frank attack of the disease or of inapparent infection, is es-sentially an antitoxic immunity. Antibacterial substances appear to be of little significance and the refractory state is associated with the presence of antitoxin in the blood serum and body fluids.

The Schick Test. Immunity to diphtheria, then, may be measured by the amount of circulating antitoxin present in a given individual. A skin test has been devised by Schick, and is known as the *Schick test,* in which a minute amount of diphtheria toxin is injected intradermally. In the non-immune the irritant action of the toxin gives rise to a local erythema followed by necrosis and desquamation, and the reaction is said to be positive. In the immune, how-ever, the toxin is neutralized by the antitoxin that is present, the characteristic reaction does not develop, and the reaction is negative. The amount of toxin injected is usually 1/50 of a guinea-pig MLD in a volume of 0.1 or 0.2 ml.; the Permanent Standards Committee of the League of Nations specifies 1/40

MLD in 0.2 ml. and 1/50 MLD in 0.1 ml.[23] According to Moloney and Taylor,[24] however, a toxin of three times this strength gives more sharply defined reactions. Considerable interest has attached to diluents for the Schick toxin since it is not stable in phenol-saline solutions. The dilute toxin is, however, stable in 2 per cent peptone solution, a borate buffer-gelatin solution, and a glycerol-gelatin solution which has been proposed recently. The advantages of a ready-diluted Schick toxin are obvious, and toxins diluted ready for use are now generally available.

For many years a negative Schick test has been regarded as indicating the presence of 1/20 unit or more of antitoxin per milliliter in the blood serum and a positive test less than 1/40 unit. More recent experiments, however, have indicated that the so-called "Schick level" of immunity is much lower than this and in the neighborhood of 1/250 to 1/500 unit of antitoxin; negative reactions have been obtained in persons with as little as 0.0005 unit. Phair[25] has expressed the opinion held by a number of workers that a negative Schick test is indicative not only of antitoxin content of the blood but also involves a defense mechanism other than that of antitoxin production.

A scarification test in which diphtheria toxin is introduced by punctate scarification rather than intradermal injection has been introduced by Reh and is called *Reh's test*. It is said to be somewhat simpler to perform than the Schick test and, when carried out with a potent toxin (with a guinea-pig MLD of 2000 per ml.), to give parallel results with the Schick test.

The question of whether the Schick test is indicative of a degree of immunity such that subsequent infection is highly improbable is one that cannot be answered *a priori*. Experience has shown, however, that the assumption that a Schick-negative person is, for all practical purposes, immune, is pragmatically sound.

Prophylactic Immunization. It was early observed that experimental animals can be immunized to diphtheria by the injection of living cultures of the bacilli after a protective dose of antitoxic serum or by the inoculation of toxin neutralized with antitoxin. Theobald Smith suggested the use of toxin-antitoxin mixtures in the immunization of horses in 1907, and the same method was used by von Behring in 1913 to immunize children. The use of toxin-antitoxin for the immunization of man, however, was developed largely through the efforts of Park in New York City from 1913 onwards.

Toxin-Antitoxin. The mixture usually used contains 0.1 L_+ dose of toxin per milliliter. The toxin is slightly underneutralized (5 ml. of the mixture should produce diphtheritic paralysis in 300 gm. guinea pigs) but depends for its immunizing efficiency not on the slight excess of toxin but on a slow dissociation of the toxin-antitoxin complex to liberate free toxin. Administered in 3 doses of 1 ml. each at intervals of one to two weeks, toxin-antitoxin produces an immunity in 85 per cent of individuals inoculated. The immunity

[23] Report of the Permanent Commission on Biological Standardization, League of Nations Health Organization. London. 1931.
[24] Moloney and Taylor: Jour. Immunol., 1937, 33:191. See also Cameron and Gibbard: Canadian Jour. Pub. Health, 1941, 32:83.
[25] Phair: Amer. Jour. Hyg., 1942, 36:283.

develops slowly, and one to six months may be required for the Schick reaction to become negative. Accidents may occur as a consequence of dissociation of the toxin-antitoxin mixture—freezing in one instance produced such dissociation—but these are rare, particularly with the 0.1 L_+ dose mixture. There is, of course, the possibility of sensitization of the inoculated individual to horse serum.

Toxoid. The use of formol toxoid or anatoxin as an immunizing agent was introduced by Ramon in 1923 and has been widely adopted. As pointed out elsewhere (p. 204), toxin treated with formaldehyde (in this case a potent toxin of more than 15 Lf doses per milliliter is incubated with 0.3 to 0.4 per cent formalin at 37° C. for one month) loses its toxicity but retains its antigenicity and is a highly efficient immunizing agent. The administration of this material in three doses of 0.5, 1.0 and 1.0 ml. at intervals of two to three weeks renders 95 per cent of persons Schick-negative. It was at first thought that toxoid might entirely replace toxin-antitoxin as an immunizing agent, but this has not proved to be the case. Reactions to the bacillary protein, while not of great importance as a rule in young children, may be relatively severe in older persons, and its use is best restricted to children under twelve years of age. Reactivity may be tested for by the intradermal injection of toxoid —the *Moloney test.*

Toxoid-antitoxin floccules (the precipitate coming down at the optimal antigen-antibody ratio) have been used in England to a considerable extent. There is, presumably, a partial purification of the toxoid by precipitation with antibody.[26] This material and toxin-antitoxin floccules have not been widely used in the United States. Toxoid precipitated with protamine appears to be an effective immunizing agent without giving the untoward reactions sometimes observed with alum-precipitated toxoid.[27] Pillemer and Toll[28] have produced highly purified toxoid by methanol precipitation in the cold which gave 2000 or more Lf per mg. nitrogen, but this material has not as yet been adequately tested as an immunizing agent.

Alum-Precipitated Toxoid. It has been found that toxoid precipitated with potassium alum (small amounts, 1 to 2 per cent, are required) is superior as an immunizing agent to ordinary formol toxoid. Present preparations are treated with charcoal prior to alum precipitation to remove color and extraneous nitrogenous material.[29] The precipitate is insoluble (it may be redissolved in sodium citrate or sodium tartrate) and remains in the subcutaneous tissue for a considerable period of time, thus providing a prolonged antigenic stimulus. It was first thought that a single injection of this material was sufficient to provide a solid immunity; some of the early reports indicated that 90 to 95 per cent of Schick-positives became Schick-negative as a consequence of a single injection. The administrative advantages of a single injection are, of course, obvious. It has become increasingly clear in recent years, however, that a single injection is not sufficient; as little as 11 per cent conversion has been

[26] For a discussion of this material see Watson, Taggart and Shaw: Jour. Path. Bact., 1941, 53:63.

[27] Ross: Amer. Jour. Dis. Children, 1944, 68:172.

[28] Pillemer and Toll: Science, 1947, 105:102.

[29] For details of preparation see Barr, Pope, Glenny and Linggood: Lancet, 1941, ii:301.

reported. There is also a tendency for such Schick-negatives to revert to Schick-positives within a year or two, though why this should be the case is not at all clear. It seems established that a single injection is not sufficient but two injections provide a solid immunity. The primary dose should be not less than half the total toxoid given and may be as great as two-thirds of it.[30] Alum toxoid has the same tendencies to produce untoward reactions in older persons that are observed with formol toxoid.

Passive Immunity. Susceptible, *i.e.*, Schick-positive, individuals may be passively immunized to diphtheria by the injection of antitoxic horse serum or purified preparations of antitoxin. Such immunity is of relatively short duration and is not effective for longer than two or three weeks at the most. Passive immunization is not so extensively practiced now as it formerly was but is, of course, indicated in the case of susceptible individuals who are directly exposed to the disease. Except on a small scale, as in a hospital ward, it is not practical to control epidemic diphtheria through passive immunization.

SUSCEPTIBILITY OF VARIOUS AGES TO DIPHTHERIA
(As Indicated by the Shick Test)

Age	Susceptible, Per Cent
Under three months	15
Three to six months	30
Six months to one year	60
One to two years	70
Two to three years	60
Three to five years	40
Five to ten years	30
Ten to twenty years	20
Over twenty years	12

The use of combined active-passive immunization in which both toxoid and antitoxin are given simultaneously, the former in protective amounts, has been of some interest. More recent work indicates that the passive protection conferred by antitoxin does not interfere seriously with the immune response though there is a period of low immunity, after the second or third week, after the passive protection has been exhausted. A second inoculation of toxoid is, of course, highly desirable.[31]

The Therapeutic Use of Antitoxin. Serum therapy in diphtheria is more successful than in any other disease, and there is no question of its efficacy in reducing the case fatality rates. As in the case of tetanus and botulism, the therapeutic administration of antitoxin cannot bring about repair of tissues already damaged by toxin. Early administration is, therefore, essential, and there is progressive increase in the case fatality rate with each day's delay. Park advises in mild cases 3000 to 5000 units, in moderately severe cases 10,000 units, and in severe toxic cases 20,000 units or more in adults and 10,000 to 20,000 in children. There is no limit, beyond the volume, to the number of units that may be safely injected. Antitoxin is generally administered intra-

---

[30] Cf. Bousfield: Brit. Med. Jour., 1943, p. 706.

[31] See Downie, Glenny, Parish, Smith and Wilson: Brit. Med. Jour., 1941, p. 717; Phair and Root: Amer. Jour. Hyg., 1942, 35:377.

muscularly but in severe cases may be given intravenously. It is completely in-effective when given by mouth.

Of considerable practical significance is the concentration of antitoxin, since the volume injected is a limiting factor. Usually horse serum contains 500 to 700 units per ml. and exceptionally 1000 to 1500. Concentration of the anti-toxin by salting out and other procedures is generally practiced, for, although some antitoxin is lost in the process, the concentration is increased with a corresponding reduction in the volume to be injected.

Epidemiology.[32] The epidemiology of diphtheria is considerably better understood than that of any other disease, in part because the causative agent can be isolated with relative facility from infected individuals, and in part
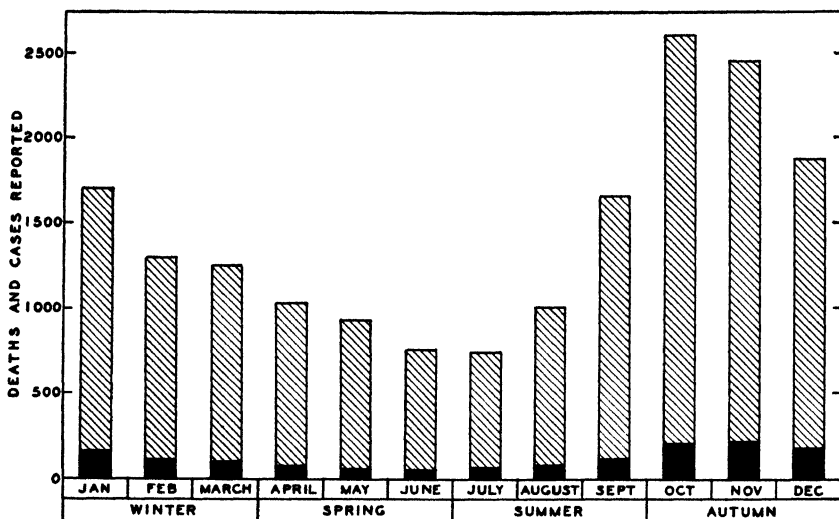
Fig. 142. The seasonal incidence of diphtheria. Averages of reported cases by months for the years 1939 to 1945 inclusive. Data from Supplements to Public Health Reports.

because the Schick test allows the differentiation of the immunes and the non-immunes. As in the case of other respiratory diseases, infectious material leaves the body in the secretions of the nose and throat, is transmitted from man to man by contact or infective droplets, and enters the body via the mouth and nose. Furthermore, the diphtheria bacillus is disseminated not only by persons with the disease but also through the agency of healthy carriers in whom there is no clinical evidence of infection. Unlike many of the diseases of the respiratory tract, however, diphtheria is an immunizing disease and prolonged or repeated contact with the bacillus frequently results in the de-velopment of a solid immunity to the disease in its clinical manifestations.

Immunity and Susceptibility. Schick testing indicates that while susceptibil-ity is low in the first six months of life, the proportion of Schick-positives in-creases rapidly and is at a maximum in children under four or five years of age, then gradually declines until 80 per cent or thereabouts of adults are

[32] For a critical discussion of the epidemiology of diphtheria during the past forty years see Russell: Med. Res. Council (Great Britain), Spec. Rept. Ser. No. 247, 1943.

Schick-negative. (See the accompanying table.) The initial immunity of the very young is passively transferred from the mother and is not of long duration. The increase in the proportion of immunes, however, is by no means entirely a result of recovery from clinical diphtheria, and the question arises as to how these individuals acquire an active immunity.

Carriers. As indicated above, healthy individuals may harbor virulent diphtheria bacilli in their throats. These carriers need be neither immunes nor convalescents and are, for the most part, casual carriers. There is no precise information concerning the duration of this transient carrier state; it may possibly be about two weeks. The proportion of carriers has been investigated by a number of workers. In a study of Baltimore school children, Doull and Fales[33] found an average carrier rate of 2.32 per cent from November to May. On the basis of this Frost[34] has estimated the carrier incidence in the five to
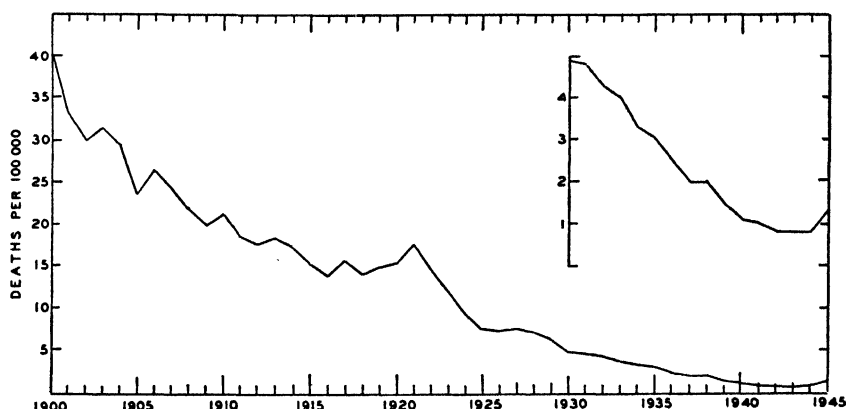
Fig. 143. The prevalence of diphtheria in the Registration Area since 1900 as indicated by the uncorrected death rates. From data compiled by the Bureau of the Census.

fourteen age group in that city to be 2538 per 10,000. At this rate 75 per cent of the population becomes infected at least once in five years, 95 per cent in ten years, and over 99 per cent in fifteen years, while very considerable proportions would suffer repeated infections, the average being 2.5 infections per person in ten years. Others have recorded considerably higher carrier rates; Dudley[35] has reported 6.6 per cent in a boys' school, and repeated swabbings showed that at least 40 per cent carried the diphtheria bacillus at one time or another during the yearly period.

There is, it appears, ample opportunity for contact with virulent diphtheria bacilli, and there is every reason to suppose that the increasing proportion of Schick-negatives in the progressively higher age groups is a consequence of an active immune response to the presence of these microorganisms in the nose and throat. It may be noted parenthetically that a similar situation may very likely prevail in certain other diseases in which technical difficulties have prevented its demonstration.

[33] Doull and Fales: Amer. Jour. Hyg., 1923, 3:604.
[34] Frost: Jour. Prev. Med., 1928, 2:325.
[35] Dudley: Jour. Hyg., 1932, 32:193.

It may be asked why contact with virulent diphtheria bacilli does not result in clinically apparent infection more often. The production of disease is, of course, dependent upon the balance between virulence and resistance as pointed out in an earlier chapter (Chap. 8). Climate appears to be of some importance, for the proportion of Schick-negatives in tropical countries is quite as high as in the temperature zones yet clinical diphtheria is much less common. Racial factors may be involved also, for in the Baltimore studies the carrier rate in Negroes was not significantly different from that in whites, yet the morbidity rate for the former was much lower.

The opportunities for the transmission of infection from person to person are reflected in both the morbidity and the mortality rates. There appears, for example, to be a direct relation between school attendance and the incidence of diphtheria, and the deaths from this disease tend to be concentrated in the preschool age group with increasing urbanization.

The Control of Diphtheria. It will be obvious from the above considerations that diphtheria is widely disseminated in the human population and cannot be controlled by the isolation of carriers or, except in a strictly limited sense, by quarantine of cases. The control of diphtheria is entirely a matter of immunization and, if a sufficiently large proportion of the susceptible population is rendered immune, the prevalence of clinical diphtheria should decrease. Godfrey[36] has found that the immunization of 50 per cent or more of the children of school age, five to fourteen years of age, did not produce a fall in the incidence of diphtheria in a number of large American cities, but that when 30 per cent or over of the preschool children were immunized there was a definite reduction in the incidence of diphtheria not only among these children but in the community as a whole. Immunization confers a marked, though not absolute, protection in the individual case, of course, and it would appear best from both the individual and group points of view that immunization be effected early.

To what extent prophylactic inoculation and the therapeutic use of antitoxin have influenced the decline in diphtheria shown in Fig. 143 is problematical. The disease was endemic during the first half of the nineteenth century though showing increasing epidemic tendencies. Between 1850 and 1860 a great pandemic developed, apparently from a focus in France, which swept over the world. A high mortality was maintained for twenty-five or thirty years, then around 1885 a decline set in which continued to about 1941. Immunization was not generally practiced until about 1920, though antitoxin therapy began somewhat earlier. Russell[32] is of the opinion that the decline in certain areas, such as New York, in which active immunization has been extensively applied is more rapid than could be expected from the trend of pre-immunization years, and attributes this increase to prophylactic inoculation.

Since 1941 there has been a general increase all over the world in the prevalence of diphtheria. It apparently began in Germany in 1939, possibly due in part to mass movement of children into camps without adequate immunization, with a doubling of the already high (285 and 207 in Austria and Germany) morbidity rate, and an increase in severity as indicated by increase in case fatality rates of 3.8 per cent in 1937–38, to 4.4 per cent in 1939, and 5.0 per cent in 1940. The disease spread into neighboring countries in northwest

[36] Godfrey: Amer. Jour. Pub. Health, 1932, 22:237.

Europe. In Belgium the number of cases rose from 2419 in 1939 to 16,072 in 1943; in Holland there were 1273 cases in 1939, 5501 in 1941, 19,527 in 1942 and 56,603 in 1943; in Norway there were 54 cases in 1939 and 22,787 in 1943. Diphtheria was the leading epidemic disease of the war years in Europe and an important cause of death in the German army. By 1945 it had declined somewhat, but the rate in the American and British occupation zones in Germany was still 430 to 560 with a case fatality rate of 5.7 per cent, and in France and Belgium the morbidity rates were still about three times the prewar rate. Some rise was also experienced in the United States, from 13,744 cases reported in 1943 to 17,612 in 1945, and in the large cities the mortality rate rose from 0.56 in 1941 to 0.88 in 1946.[37] The sharply increased prevalence of a more severe diphtheria has led to some speculation as to the possible appearance of unusually virulent strains but there has been no bacteriological evidence in support of the suggestion. It seems more likely that the increased prevalence is attributable to some degree of breakdown in the application of artificial immunization, due in part to apathy and in part to population dislocations coincident with war, but in general it has not been adequately explained. Such a phenomenon serves as a timely reminder that, though many of the infectious diseases have been brought under some degree of control, that control must continue to be exerted.

THE DIPHTHEROID BACILLI

Microorganisms morphologically closely similar to and frequently indistinguishable from the diphtheria bacillus, known as diphtheroid bacilli, are found in man and lower animals. Three species are commonly observed in man. A form occurring in the human throat and readily confused with the diphtheria bacillus on microscopic examination was first observed by Löffler and by von Hofmann-Wellenhof. It is known as *Hofmann's bacillus, Corynebacterium hofmannii,* or *Corynebacterium pseudodiphtheriticum.* It differs slightly from the diphtheria bacillus in that it is somewhat shorter and plumper and does not ferment dextrose. Most important, it does not form a soluble toxin and is readily differentiable from *C. diphtheriae* by the virulence test. It seems to be completely non-pathogenic for man and experimental animals.

A second species, *Corynebacterium xerose,* has been isolated repeatedly from a form of conjunctivitis known as xerosis, but its etiologic relation to the disease is highly uncertain. It is also found on the skin where it is presumably a part of the normal bacterial flora, and it is probable that its presence in xerosis is that of a contaminant. It does not form a soluble toxin. *Corynebacterium acnes* has been found with some frequency in acne pustules but whether the association is causal is open to serious question. This organism stands somewhat apart from the other bacilli of the group in that it is micro-aerophilic and grows profusely under anaerobic conditions with the formation of a pink pigment. It also does not form a soluble toxin.

A number of species of corynebacteria are pathogenic for lower animals and rarely may infect man. *Corynebacterium pyogenes* is one of the commonest causes of purulent infections in cattle, sheep, pigs and goats; it is the cause of a form of mastitis, a few cases of abortion, arthritis and granulomatous

[37] For references and data see Stowman: Epidemiological Info. Bull. (UNRRA), 1945, 1:157; *ibid.*, 1946, 2:147; Anderson: Amer. Jour. Pub. Health, 1947, 37:1.

lesions in bovines, and is associated with calf pneumonia as well. Infections of man have been reported.[38] It forms a soluble toxin, immunologically distinct from and considerably weaker than that of the diphtheria bacillus, which is hemolytic for rabbit erythrocytes, lethal for mice, and produces a dermal necrosis in the rabbit similar to that produced by diphtheria bacillus toxin. This bacterium and its toxin have been studied extensively by Lovell.[39]

Corynebacterium ovis (*Corynebacterium pseudotuberculosis*) or the Preisz-Nocard bacillus is also a not uncommon pathogen of domestic animals. It produces a caseous lymphadenitis and ulcerative lymphangitis in sheep and horses referred to as pseudotuberculosis, and ulcerative lesions in other domestic animals. Like *C. pyogenes,* it forms a weak exotoxin distinct from diphtheria toxin. *Corynebacterium renale* is closely related serologically to *C. ovis* and produces purulent infections of the urinary tract in cattle, sheep, horses and
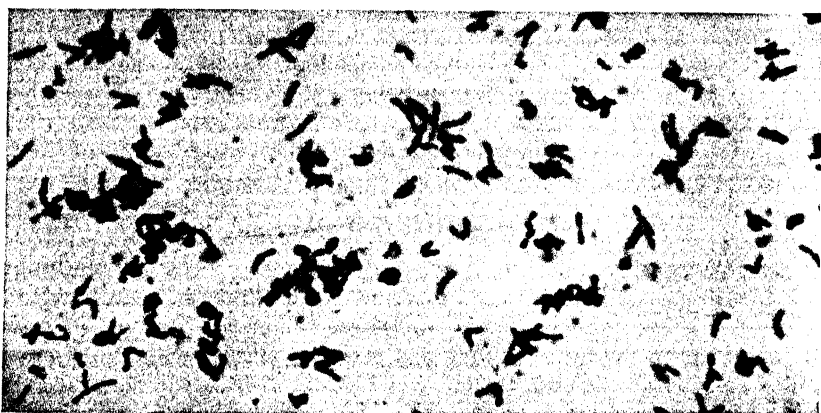
Fig. 144. *Corynebacterium pseudodiphtheriticum;* smear from pure culture stained with alkaline methylene blue. Note the irregular staining, club-shaped forms, and general close resemblance to *C. diphtheriae.* × 1050.

dogs. *Corynebacterium equi* is the cause of a spontaneous pneumonia in foals and other infections in horses; this species is of interest in that it is variable in its reaction to the acid-fast stain, the coccoid forms retaining the stain while the bacillary forms take the counterstain, suggesting a relationship to the mycobacteria and acid-fast actinomycetes. *Corynebacterium enzymicum* has been isolated from man primarily, but has been found as the cause of an epidemic ophthalmia of sheep.[40] *Corynebacterium murisepticum* is the cause of a mouse septicemia and is apparently pathogenic for no other animals. The occurrence of these and other diphtheroid bacilli in diseases of domestic animals makes this group of microorganisms of considerable interest in veterinary medicine.

In addition to these a dozen or more authenticated species of corynebacteria are soil saprophytes or pathogenic for plants, producing diseases of wheat, alfalfa, ring rot of potato, bacterial canker of tomato and poinsettia, bean wilt, and the like.

[38] *Cf.* Ballard, Upsher and Seely: Amer. Jour. Clin. Path., 1947, 17:209.
[39] Lovell: Jour. Path. Bact., 1937, 45:339; *ibid.,* 1939, 49:329; *ibid.,* 1941, 52:295; *ibid.,* 1944, 56:525; Vet. Record, 1945, 57:386.
[40] Bruce: Canadian Jour. Comp. Med., 1943, 7:369.

MYCOBACTERIUM

This genus includes a number of species of related bacteria which are most conveniently considered in three groups. The first includes the mammalian tubercle bacilli, *Mycobacterium tuberculosis* var. *hominis* and *Mycobacterium tuberculosis* var. *bovis,* and the avian tubercle bacillus, *Mycobacterium avium.* In the second group there are Hansen's bacillus or *Mycobacterium leprae,* and the rat leprosy bacillus, *Mycobacterium leprae murium.* The third group is made up of Johne's bacillus, or *Mycobacterium paratuberculosis,* and certain acid-fast bacilli isolated from cold-blooded animals, together with the saprophytic acid-fast forms.

THE TUBERCLE BACILLI

Tuberculosis is an old disease of man and is still one of the most widespread; about 75,000 persons die of tuberculosis in the United States each year. Its infectious nature was suspected by Fracastorius in the early part of the sixteenth century, and Villemin showed, in 1865, that the disease could be transmitted by the inoculation of tuberculous material. It was in 1882 that Koch demonstrated the tubercle bacillus by special staining methods, isolated and grew it in pure culture, and reproduced the disease by the inoculation of the bacilli.

Morphology and Staining. The tubercle bacilli are slender, sometimes slightly curved rods 2 to 4 μ in length and 0.3 to 1.5 μ in breadth. They occur singly but are often found in small groups, sometimes in compact masses in which the individual bacilli cannot be distinguished. The bacilli of the human variety tend to be somewhat longer and more slender than those of the bovine type, but the morphology of both is variable and no distinction can be made on this basis. The bacillary form is generally retained in the tissues; in culture longer filamentous forms are sometimes seen together with swollen or club-shaped cells resembling the diphtheria bacillus. Branched forms are present in cultures of the avian tubercle bacillus but are rarely seen in cultures of the mammalian bacilli. The occurrence of filamentous forms and true branching indicates the close relation of these bacilli to the higher fungi; hence the name *Mycobacterium* and the placing of these microorganisms, together with the diphtheria bacillus, in the order Actinomycetales.

The tubercle bacillus is non-motile and non-spore-forming and produces a capsular substance in artificial cultures, particularly when grown upon serum media. The granular structure of the individual cells is marked. Vacuoles often occur in abundance and may even give the stained cell the appearance of a chain of cocci. The significance of the small, deeply staining bodies sometimes observed within the cells is not clear; they do not exhibit the enhanced resistance characteristic of spores.

The tubercle bacilli cannot be stained by the usual staining methods that are effective with other bacteria, for there is a marked resistance to the penetration of dyes into the cell that is associated with the presence of relatively large amounts of unsaponifiable wax. The cells may, however, be stained in two or three minutes by steaming carbol fuchsin or by prolonged (twenty-four to thirty-six hours) exposure to the dye at room temperature. Once stained, the bacilli are difficult to decolorize and resist the action of alcohol and dilute solutions of mineral acids and for that reason are termed "acid-fast." They may be demonstrated in smears by the Ziehl-Neelsen method, in which the smear is stained with hot carbol fuchsin, decolorized with acid alcohol, and counterstained with a dye of contrasting color. Methylene blue is most commonly used but some workers prefer other stains such as picric acid, Bismarck brown, etc. Non-acid-fast bacilli may be observed in young cultures.
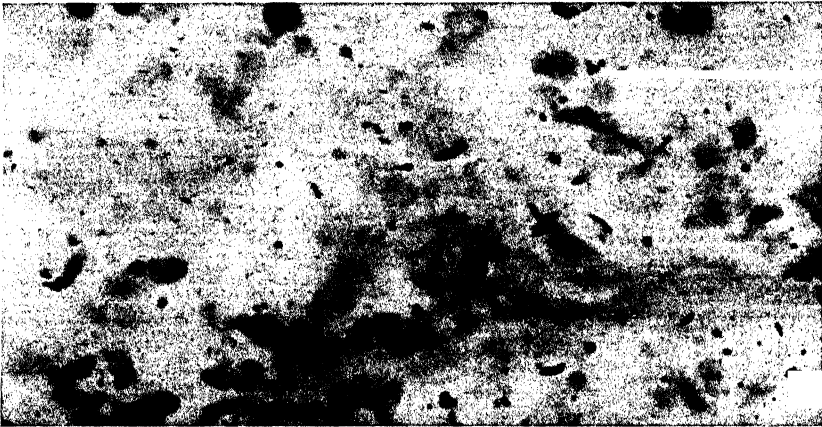
Fig. 145. *Mycobacterium tuberculosis.* Acid-fast stained smear of tuberculous sputum. × 1050.

More recently fluorescent microscopy has been applied in the detection of tubercle bacilli in sputum smears, concentrates and similar material. The smear is stained with carbol-auramine, a solution of auramine in 3 per cent phenol, decolorized with acid alcohol, and examined in ultraviolet light. There is no counterstain. The ultraviolet irradiation need not be intense and a brilliant filament lamp with a blue ultraviolet-transmitting filter and an aluminum mirror suffice for use with the ordinary microscope.[1] The tubercle bacilli retain the dye which fluoresces in ultraviolet light, and appear as brilliant yellow bacilli on a dark background. The use of cresol in concentration of the bacilli interferes with the examination, for it fluoresces also. This staining method may also be used for tissue sections. Auramine is retained by the tubercle bacillus in the same way that fuchsin is retained, *i.e.,* by virtue of the presence of the acid-fast wax mycolic acid, and bacilli rendered non-acid-fast by treatment with organic solvents no longer retain auramine.

[1] For detailed discussions of the method and its application see Richards, Kline and Leach: Amer. Rev. Tuberc., 1941, *44*:255; Bogen: *ibid.,* 1941, *44*:267; Lind and Shaughnessy: Jour. Lab. Clin. Med., 1942, 27:531.

The tubercle bacilli are gram-positive. Aniline gentian violet must be applied warm for two or three minutes. It has been suggested that the iodine solution plays no part in the retention of the stain as in the case of other gram-positive bacteria and the failure to decolorize is a consequence of the acid-fast nature of the cells.

Non-acid-fast but gram-positive granules, known as *Much granules,* were described by Much in 1907 as occurring in the material from cold abscesses and elsewhere in which acid-fast bacilli could not be demonstrated but which, nevertheless, proved to be infective. Considerable numbers of acid-fast bacilli, perhaps 100,000 per milliliter, must be present, however, before there is a reasonable chance of finding them in smears. Much maintained that these granules are viable and virulent and give rise to typical acid-fast rods. They have been observed by others but their significance is open to question. Some
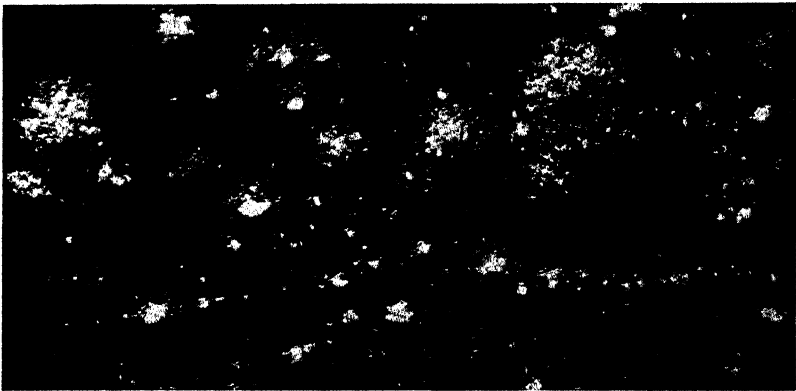
Fig. 146. Colonies of the human variety of the tubercle bacillus, H-37 strain, on Lowenstein's medium, five weeks' incubation. × 3.

workers regard them as degeneration products or artifacts of the staining procedure.[2]

In broth cultures there is a thick, wrinkled skin of surface growth which tends to spread up the sides of the flask; masses of bacilli may become detached and sink to the bottom as a lumpy sediment. Growth on the surface of solid media is generally dry and granular with nodular, heaped-up areas. The human variety of the tubercle bacillus usually produces a pale yellow or orange-yellow growth on serum-containing media and a creamy or white growth in the absence of serum. The bovine variety is not pigmented on serum media. Some avian strains give a faint pink-colored growth on egg media. A peculiar almond-like odor is often noticeable in cultures of these bacteria.

Physiology. The tubercle bacillus is an aerobe and will not grow under completely anaerobic conditions. The mammalian varieties grow best at 37° C. and not at all below 30° C. or above 42° C.; the optimum temperature for the avian type, however, is 40° C. Growth is relatively slow, and four to six weeks are generally required for an abundant growth, although minute colonies appear in eight to ten days. Most strains of the avian type adapt them-

[2] *Cf.* Porter and Yegian: Jour. Bact., 1945, 50:563.

selves readily to culture on artificial media and in time are able to grow much more rapidly, but others remain slow-growing.

The tubercle bacillus is difficult to cultivate upon primary isolation. Blood serum, coagulated by inspissation, was used by Koch, and this medium still remains one of the most satisfactory. Media containing eggs, either the yolk or yolk and white combined, glycerol, and sometimes dyes, are also used for primary isolation. Dorset's egg medium is simply whole egg mixed with a little water and coagulated in slant form by inspissation. Petroff's medium, one which is widely used, consists of an infusion base to which eggs, glycerol and gentian violet have been added, and is sterilized by inspissation. Lowenstein's medium is more complex and contains egg, potato meal, bone marrow infusion, citrate, glycerol and asparagin. Glycerolated potato is used by the French workers. Corper's medium is glycerolated potato with the modification that the pieces of potato are soaked for a short time in a solution of crystal violet before sterilization with the glycerol solution. The dye in this and Petroff's medium serves to inhibit the growth of contaminating bacteria.

The human variety of the tubercle bacillus grows more abundantly on all of these media than does the bovine variety, and for that reason it is termed "eugonic" and the bovine type "dysgonic." These two varieties also differ in that glycerol is markedly favorable to the growth of the human type but does not so affect the bovine type. It is not known why glycerol exerts this favorable effect; attempts to substitute related compounds such as isopropyl alcohol, propyl alcohol, glycol, trimethylene glycol and inositol have not been successful. Glucose, however, acts in much the same manner as glycerol. Egg-yolk has been reported to contain a lipid growth factor, but this appears to stimulate rather than be essential to growth. Thiamine, pyridoxine and riboflavin do not stimulate growth.

Growth occurs much more readily and upon simpler media after primary isolation. The human tubercle bacillus grows well upon nutrient agar or broth containing glycerol (2 to 5 per cent) and has been cultivated in a variety of synthetic solutions. One of the best known of these is Long's synthetic medium which contains glycerol, asparagin, citrate and inorganic salts. Dubos and his co-workers[3] have found that growth is facilitated and occurs diffusely throughout the liquid medium in the presence of certain water-soluble lipids. A medium containing asparagin, glucose, phosphate, citrate, bovine serum albumin, magnesium sulfate and a lipid commercially designated Tween 80 (a polyoxyethylene derivative of sorbitan mono-oleate), will give visible growth of the human variety of tubercle bacillus within two weeks. The medium becomes inhibitory in time owing to the activity of lipase contaminating bovine albumin preparations, which liberates oleic acid to bacteriostatic concentrations from the Tween 80; the effect can be eliminated by the use of commercial crystalline bovine serum albumin, the addition of 0.01 per cent sodium fluoride, etc. The bovine type grows poorly or not at all on these media and is benefited only slightly by the presence of glycerol. The avian type of tubercle bacillus, however, grows better than the human type after a few transfers and good growth may be obtained on nutrient agar in the absence of glycerol. Like the human type, however, the avian bacillus grows much more profusely in the

[3] Dubos: Proc. Soc. Exp. Biol. Med., 1945, 58:361; Dubos and Davis: Jour. Exp. Med., 1946, 83:409; Dubos and Middlebrook: Amer. Rev. Tuberc., 1947, 56:334.

presence of glycerol. These cultural differences in the three types of tubercle bacilli are of little significance in their early differentiation, for several transfers over a period of months are necessary before they are obvious. They are summarized in the table on p. 642.

The biochemical reactions of the tubercle bacilli have not been studied at length. Growth occurs in milk, but no visible change is produced. Indol is said not to be formed. No acidity is developed in sugar broths, but it has been found by analytical methods that dextrose, arabinose and sometimes sucrose are utilized while lactose is not. Whether the oxidation proceeds to completion or whether formed acids are neutralized by liberated ammonia is not known. In glycerol broth, cultures of the bovine type become alkaline while those of the human type become slightly acid. It has been reported that the source of alkali is the nitrogenous constituents of the medium, and acid is produced from glycerol but is further oxidized. All intermediate gradations occur, however, and no differentiation between the two types is possible on this basis.

Chemical Composition. The chemical composition of the tubercle bacilli has been more intensively investigated than that of any other bacteria. These bacilli are of particular interest in this connection because of their high content of lipoidal substances which may make up as much as 40 per cent of the dry weight. Protein, a considerable proportion of which is nucleoprotein, makes up about half the dry weight and polysaccharides are found in relatively small amount.[4]

The lipids have been studied at length by Anderson[5] and his co-workers. In addition to neutral fat, two general types of material may be distinguished:

(*a*) Phospholipid, containing saturated and unsaturated fatty acids including the well-known palmitic, linoleic and linolenic acids, together with two acids peculiar to the tubercle bacillus, *phthoic acid,* isomeric with cerotic acid, and *tuberculostearic acid,* isomeric with stearic acid and optically inactive.

(*b*) An acid-fast wax, containing polysaccharides hydrolyzing to mannose, arabinose and galactose; a soft wax which is a complex glyceride; and an unsaponifiable wax (acid-fast) made up of higher alcohols and including a high molecular weight saturated hydroxymethoxy acid termed *mycolic acid,* a higher alcohol designated *phthiocerol,* and a levorotatory fatty acid, *mycocerosic acid.*

Some of these substances appear to be physiologically active. The unsaponifiable acid-fast wax apparently stimulates the multiplication of undifferentiated connective tissue cells, and phthoic acid induces a proliferation of epithelioid cells.[6] Whether they are immunologically active is not clear. It may be noted that a yellow pigment is found in the neutral fat which is designated *phthiocol* and is a hydroxynaphthaquinone which may be reversibly reduced at a relatively low potential.

Polysaccharide mixtures, containing immunologically active and inactive substances, have been isolated from mammalian tubercle bacilli[7] but their significance is not as yet understood. The protein constituents of the cell appear

[4] See the review of the protein constituents of the tubercle bacillus by Seibert: Bact. Rev., 1941, 5:69.

[5] *Cf.* Anderson: Physiol. Rev., 1932; 12:166; Chem. Rev., 1941, 29:225.

[6] *Cf.* Sabin: Physiol. Rev., 1932, 12:141; Amer. Rev. Tuberc., 1941, 44:415.

[7] See Menzel and Heidelberger: Jour. Biol. Chem., 1939, 127:221.

to be the most important immunologically and have been studied in connection with the preparation and activity of the various tuberculins which are considered in a later section.

Resistance. Although exhibiting much the same degree of resistance to heat as the vegetative cells of other bacteria, the tubercle bacilli are relatively highly resistant to drying, chemical disinfectants and other deleterious environmental influences, very likely as a consequence of their content of wax. In putrefying sputum the bacilli may remain viable for weeks or months and in dried sputum kept in a cool dark place for as long as six to eight months. Sputum that is completely dried, so that particles are capable of floating as dust in the air, may be infective for eight to ten days.[8] In dried sputum they may survive 100° C. for an hour but are killed in the usual way by moist heat. Phenol penetrates the bacilli only slowly and a 5 per cent solution requires twenty-four hours to kill the bacilli in sputum. The action of other disinfectants is similarly retarded, and it may be noted that the hypochlorites have almost no effect on these bacteria. Tubercle bacilli, however, are readily killed by exposure to direct sunlight; bacilli from cultures are killed within two hours but in sputum may survive twenty to thirty hours of such direct exposure.

Variation. The variability of the tubercle bacilli has been studied extensively, particularly in recent years, but with inconclusive results. Colonial variants, thought by some to be analogous to the S and R variants of other bacteria, have been observed, and it has been claimed by some workers that the S type is the more virulent, and by others that virulence and colonial morphology are independent. It may be noted that the colonial morphology of the tubercle bacilli is, to a considerable degree, a transient adaptation to environmental conditions and prompt alteration of colonial appearance results on transfer to a different medium. For example, Steenken[9] has observed that colonies growing in the presence of ether extract of egg yolk are smooth and markedly different from the usual colonial type, but the effect is only a temporary physical one. The status of the S-R variation in the tubercle bacilli is, then, by no means clear as yet.

Bacille Calmette-Guérin. A bovine strain of the tubercle bacillus was rendered completely avirulent by Calmette,[10] who cultivated it over a long period of time (230 transfers in thirteen years) on bile-glycerol-potato medium. This strain is designated as BCG (*Bacille Calmette-Guérin*) and has been of particular interest in connection with active immunization against tuberculosis. The nature of the change which resulted in the loss of virulence is completely unknown. The loss appears to be permanent and virulence does not reappear on transfer to ordinary media; Petroff,[11] however, has separated "rough" and "smooth" variants of BCG, and the "smooth" type proved virulent for guinea pigs.

Life Cycles. The pleomorphic tendencies of the tubercle bacilli, coupled

[8] For example, see Smith: Amer. Rev. Tuberc., 1942, 45:334.

[9] Steenken: Amer. Rev. Tuberc., 1940, 42:422.

[10] *Cf.* Calmette: *Tubercle Bacillus Infection and Tuberculosis in Man and Animals* (transl.). Williams & Wilkins Company, Baltimore. 1923.

[11] Petroff: Proc. Soc. Exp. Biol. Med., 1927, 24:632; Petroff, Branch and Steenken: *ibid.*, 1927, 25:14.

with the occurrence of non-acid-fast rods in young cultures and the granular elements described by Much, have been interpreted by a number of workers as indicative of a cyclic succession of morphologic types, or life cycle, through which these bacilli go. As pointed out in an earlier chapter (Chap. 6), the evidence for such developmental cycles is not definitive but rather a matter of interpretation. Whether swollen, misshapen cells, filamentous or branching forms and granular elements, such as those described by Much, are to be regarded as sexually reproducing forms, gonidia and the like, or as products of degenerative changes is as yet purely a matter of opinion. It may be noted that the objective evidence of micromotion pictures does not support the assumption of a complex life cycle in the development of the tubercle bacilli.[12]

Filterable Forms. Associated by some with the concept of life cycles is that of the existence of tubercle bacilli in a filterable form or "ultravirus." There seems to be little doubt that some cultures or preparations of tubercle bacilli contain forms that may pass diatomaceous earth or unglazed porcelain filters, but whether these are dwarfed bacilli or fragments of bacilli with the power to regenerate, or whether they represent a distinct "phase" in the life history of the microorganisms has yet to be determined.

Pathogenicity for Man. In the United States in 1945 there were 106,716 cases of all forms of tuberculosis, and of these 65,843 were cases of pulmonary tuberculosis. Tuberculosis is among the first three leading causes of death in a relatively large portion of the life span (15 to 49) and holds first place in the 15–34 age group, second in the 35–39 age group, and third in the 40–49 group. In 1939–41 it accounted for 10.4 per cent of the white male, 22.9 per cent of the white female, 28.8 per cent of the non-white male and 37.8 per cent of the non-white female deaths.[13] Tuberculosis is clearly one of the great killing diseases of mankind.

The mammalian tubercle bacilli, both bovine and human varieties, are pathogenic for man. The human type is practically always responsible for pulmonary tuberculosis in adults and is usually found in children also. The bovine variety may occur occasionally in pulmonary tuberculosis in children but is more often found in infections of other tissues. More than half of the cases of cervical adenitis and abdominal tuberculosis in children are infections with the bovine tubercle bacillus, and it has been estimated (1910) that this type was responsible for 6 to 10 per cent of the deaths from tuberculosis in young children; the percentage is probably lower now as a result of decreased incidence of tuberculosis in cattle in this country. Bovine tuberculosis is, then, a slight, possibly a negligible, factor in adults but is a serious matter in children under five years of age. Mixed infections with the two types of tubercle bacilli have been reported but are rare.

The avian tubercle bacillus is, for all practical purposes, non-pathogenic for man, although a few instances of human infection, including pulmonary tuberculosis, have been reported. It has been supposed by some that Hodgkin's disease, a granulomatous inflammation of the lymphadenoid tissues of the body, is an infection with avian tubercle bacilli, but this appears not to be true.

Routes of Infection. The tubercle bacillus may enter the body by way of

[12] Wyckoff: Amer. Rev. Tuberc., 1934, 29:389.
[13] Yerushalmy, Hilleboe and Palmer: Pub. Health Repts., 1943, 58:1457.

the genito-urinary tract, the conjunctiva, the skin, the alimentary tract and the respiratory tract. Primary infection of the genito-urinary tract is possible but, under natural conditions, rarely occurs. Infection through the conjunctiva takes place readily under experimental conditions; its frequency under natural conditions is not known, for the cervical lymph glands, where the infection would first appear, are readily infected by other channels. Infection through the skin is relatively rare; whether the bacilli can penetrate the intact skin is uncertain, but they may enter through abrasions or other traumatic injuries. Primary infection of the skin generally results in verruca tuberculosa (pathologist's wart) or lupus vulgaris.

Primary infection by the alimentary tract is a consequence of the ingestion of tubercle bacilli in infected food, most commonly milk, and occurs with con-
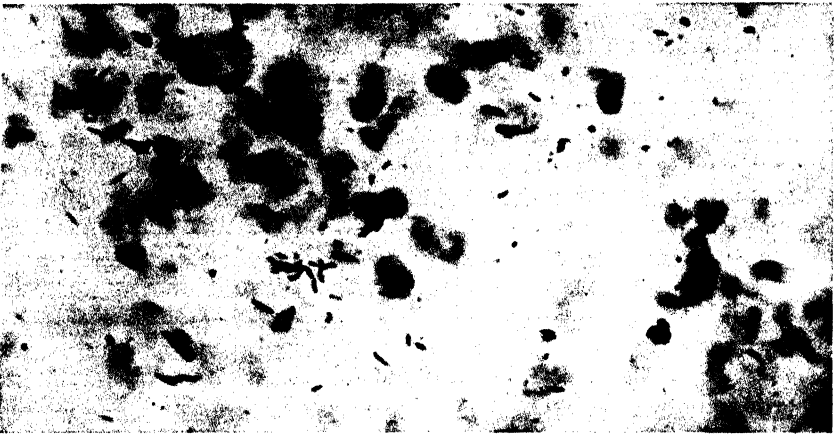
Fig. 147. Tubercle bacillus. Acid-fast stained smear of pus from a liver abscess in a rhesus monkey. × 1050.

siderable frequency in children. Secondary infection may occur, especially in children, by swallowing tuberculous material of respiratory origin. In the upper portion of the alimentary tract the bacilli enter the body tissues through the faucial, pharyngeal and lingual lymphoid follicles and first affect the upper cervical and retro-pharyngeal lymph glands. Tuberculous lesions of the tonsils, it may be noted, are not infrequent though rarely conspicuous. The stomach is rarely a portal of entry, but the bacilli penetrate the intestinal mucosa by way of Peyer's patches.

The respiratory tract is the most frequent and most important route of infection with the tubercle bacillus. The coarser infectious particles in inspired air are filtered out and deposited on the nasal, buccal and pharyngeal surfaces and the bacilli, upon penetration, set up focal infections in the local lymphatic tissue. Fine droplets or particles of dust, however, may, and frequently do, enter the lungs directly.

The Spread of Infection in the Body. Tubercle bacilli are disseminated through the body from primary or secondary foci of infection by way of the lymph, the blood stream, or directly by extension along contiguous surfaces.

Distribution via the lymphatics occurs more readily in children than in adults, and the bacilli may localize at almost any point but most commonly in the lymph nodes. Bacilli present in the thoracic duct may, of course, gain entrance to the blood stream. The blood stream may also be invaded directly through erosion of a vessel wall by a focus of infection. The bacilli are transported throughout the body by the blood and give rise to acute miliary or chronic disseminated tuberculosis. Spread by extension occurs most frequently in ulcerative pulmonary tuberculosis with the breaking down of foci of infection and the consequent presence of bacilli in the sputum. The pleura and pericardium may be invaded directly from a focus of infection in the lungs. The infection of such serous surfaces may be localized and fibrinous and result in adhesions, or may assume an acute miliary form. Direct extension may also occur elsewhere, as from the kidneys to the ureters and bladder, or into the peritoneal cavity and adjacent areas from intestinal ulcers.

It will be clear that practically every organ and tissue of the body may be invaded by the tubercle bacillus. Not all are invaded with equal frequency, however. Tuberculosis in man is most commonly the pulmonary form with a primary invasion of the apices, and over 90 per cent of the deaths from tuberculosis are due to the pulmonary type. Whether primary infection is a direct consequence of inhalation as believed by Koch, or whether it is in most cases of hematogenous origin following a preliminary infection of the lymphatic system as urged by Calmette, is not altogether clear, but on the whole the evidence favors inhalation infection. Although pulmonary tuberculosis is by far the most common form of infection in adults, it is somewhat less frequent in children, but precisely how much less is not known with certainty. Pulmonary infection in children differs from that in adults in that the hilum glands are involved. Involvement of the lymphatics is frequent in children. The anatomical distribution of the lesions usually takes one or the other of two well-defined forms; in the one the lesions are found predominantly in the tracheobronchial lymphatics, and in the other in the mesenteric lymph nodes. In general, children show a tendency toward generalized infection. In 1945 92 per cent of deaths were from respiratory tuberculosis, and the remaining 8 per cent from other forms of the disease.

Other tissues and organs are involved less frequently. The spleen, liver and kidneys are sometimes infected. Tuberculosis of the adrenals gives rise to Addison's disease. Infection of the skin or lupus is not uncommon. Bone and joint tuberculosis is more common in children than in adults, and tuberculous meningitis is not infrequent in the young. It will be clear that tuberculous infection may take a variety of clinical forms.

The Tubercle. Lesions caused by the tubercle bacillus, in whatever part of the body they occur, usually possess a definite although not absolutely characteristic appearance and histological structure. Small nodules or tubercles, plainly visible to the naked eye, are so uniformly observed in all advanced infections with the tubercle bacillus that their presence has given the name to the disease. The young tubercle probably originates from the fixed cells surrounding the invading bacilli. By the proliferation of the fixed cells, elongated "epithelioid" cells are developed in more or less definite concentric layers and come to form the substance of the tubercle. So-called "giant cells" or "foreign-

body giant cells" soon appear in the developing tubercle which are huge multi-nuclear masses of protoplasm thought by some to be especially distinctive of true tubercle formation, though it is doubtful if this criterion can be maintained. Either they are produced by the fusion of a number of macrophages or they are of single-cell origin. While the formation of epithelioid and giant cells is going on, leucocytes, at first polymorphonuclear leucocytes and later lymphocytes, cluster around the periphery of the tubercle. Degeneration of the tubercle eventually sets in, the central portion becomes necrotic, and this is followed by caseation and then by softening of the caseous mass.

In some cases calcium salts are deposited in the tubercle (calcification), converting it into a hard, dry, friable body which may become encapsulated and completely walled off from the surrounding tissues. In other instances, however, this healing process does not take place, and there is instead an extension with coalescence and the formation of confluent masses that may reach a diameter of 4 or 5 cm. The erosion of a blood vessel may occur, and the discharge of large numbers of the bacilli into the blood stream leads to a general diffusion of small tubercles of the size of millet seeds (acute miliary tuberculosis).

The early stages of tubercle formation, characterized by cell proliferation and leucocytic infiltration, are probably a response to a chemical or mechanical stimulus caused by the presence of the bacilli; the later changes, leading to necrosis and caseation, may be attributed to the action of the bacterial products. The host becomes sensitized to the bacillary substance and the allergic response of the tissues is involved to no small extent.

Predisposing Factors. Few diseases are as completely dominated by predisposing factors as tuberculosis. Infection with the tubercle bacillus is exceedingly common, and there are few adults, particularly those living in cities, who escape. The proportion of individuals coming to autopsy who show evidence of infection is very high and has been reported as high as 97 per cent. Of the more recent reports, for example, Sweany, Levinson and Stadnichenko[14] have found 25 per cent of ten-year-olds, 55 per cent of twenty-year-olds, 80 per cent of thirty-year-olds, and over 90 per cent of individuals over fifty years of age show calcified lesions of infection. The bacilli, then, are present in the great majority of adults and also in many children, but the development of clinical tuberculosis is restrained by the non-specific resistance of the host. Predisposing factors, therefore, are those which tend to interfere with normal physiological well-being and include such things as insufficient or unsuitable food, prolonged exposure to dampness, a sedentary life, chronic fatigue, etc. In the first World War the incidence of tuberculosis rose in this (see Fig. 150) and other countries, and in the second World War the same tendency was again apparent in England.[15] In both man and animals tuberculosis is a disease associated with confinement, of men in houses and cattle kept in stables. The opportunities for transmission of the infection are, of course, much greater in community life.

There is a marked occupational predisposition to tuberculosis in the dusty trades, and the constant inhalation of almost any kind of dust results in an

---

[14] Sweany, Levinson and Stadnichenko: Amer. Rev. Tuberc., 1943, 48:131.
[15] Med. Res. Council (Great Britain), Spec. Rept. Ser., No. 246, 1942.

increased incidence of pulmonary tuberculosis. Silica dusts, however, appear almost specifically to predispose to tuberculosis and the incidence of infection in those constantly exposed to such dusts is much higher than that in the general population.

It has long been suspected that there are familial or hereditary tendencies to tuberculosis in man. Resistance to infection in experimental animals is to some degree genetically determined, as pointed out elsewhere (p. 217), but the conclusive demonstration of a similar phenomenon in man is difficult owing to both a long generation time and the inability to carry out appropriate breeding experiments. The incidence of new infection in tuberculous families is, of course, considerably greater than in the general population, but whether this is a consequence of increased risk alone or in part of genetically determined factors is not clear. In any case the disease itself is not inherited and congenital tuberculosis rarely if ever occurs.

The relation of childhood infection to clinical tuberculosis in the adult has been a matter of considerable interest, and it has been suggested that pulmonary tuberculosis of the young adult may, in many instances, be a consequence of the lighting up of an old infection. The evidence, however, is against this view, and it is probable that tuberculosis in the adult is a consequence of reinfection rather than of a flaring up of the healed or partially healed lesions from childhood infection. It is to be noted, however, that a considerable period of time, possibly years in some instances, may elapse between reinfection and the appearance of tuberculosis in clinical form.

Bacteriological Diagnosis of Tuberculosis.[16] Tubercle bacilli are discharged from the infected individual in sputum and urine and may be demonstrated in these materials, and in gastric washings, spinal fluid or infected tissues, depending on the location of the infection. Of specimens from these sources, those obtained by gastric lavage are of particular value in respiratory tuberculosis in children, who tend to swallow sputum, and are also useful in adult infections. Results must be interpreted with caution because of the presence of saprophytic acid-fast bacilli in the gastro-intestinal tract derived from the ingestion of foods, especially fruit, on which these forms occur; furthermore, there is some reason to believe that some fats and oils may impart acid-fast properties to ordinarily non-acid-fast bacteria present in the alimentary tract. The presence of the bacilli may be shown either directly or after concentration by examination of stained smears, culture and guinea pig inoculation.

As a rule, some method of concentration is desirable, for not less than 100,-000 bacilli per ml. must be present before there is a reasonable chance of finding them by microscopic examination. Because of the unusual resistance of the tubercle bacillus, the infected material can be treated with an equal volume of 3 per cent sodium hydroxide, or with antiformin (a proprietary preparation of hypochlorite) in final concentration of 15 per cent for fifteen to thirty minutes without killing them. The digested material is neutralized in the case of alkali treatment, centrifuged, and the sediment used for the preparation of smears and to inoculate guinea pigs and culture media. Chemical flocculation

[16] For the minimum laboratory standards set up by the American Trudeau Society, given in technical detail, see Amer. Rev. Tuberc., 1942, 45:103.

22

with alum or ferric chloride is desirable with specimens such as urine or spinal fluid, and may be applied to digested material to facilitate spinning out of the bacilli. A flotation method of concentration may be used which consists of dilution of the digested material with water and shaking with a hydrocarbon such as xylol or gasoline; the creamy layer separating out on standing is smeared and stained.

Smears are usually stained by the Ziehl-Neelsen method; fluorescent microscopy is of sufficiently recent development that it is not yet widely used though it may well become generally adopted. The demonstration of acid-fast bacilli allows a provisional diagnosis but does not indicate whether the bacilli were viable, or whether they are virulent tubercle bacilli. Their presence in urine specimens in particular must be interpreted with caution because of the frequent occurrence of the smegma bacillus which is also acid-fast.

Guinea pigs are inoculated in the groin or in the muscle of the thigh. If a reasonable number of tubercle bacilli have been inoculated, enlargement of the regional lymphatics may be noted in two or three weeks, the pig becomes emaciated by four to six weeks and usually dies not long after. With very small numbers of bacilli evidence of the infection may be delayed two or three weeks longer. If it is not obviously ill or does not die, the animal should be kept for eight weeks before sacrificing. At autopsy the regional lymph nodes will be found to be enlarged and filled with caseous material. Necrotic areas in the spleen and liver are characteristic of the gross pathology in this animal; tubercles are seldom seen, and the lungs are only slightly affected and the kidneys almost never. Tubercle bacilli may be cultured from the lesions and found in acid-fast-stained smears. Some workers tuberculin-test the animals before inoculation and three to four weeks afterward, the development of a hypersensitivity indicating infection. If differentiation of the human and bovine varieties of the bacillus is desired, rabbits may be inoculated.

The culture of tubercle bacilli, carried out concurrently with animal inoculation, is becoming quite general and some workers feel that it is as sensitive as guinea pig inoculation. As indicated earlier, a variety of media, containing glycerol and egg, may be used, the particular medium varying from one laboratory to another. A slant is inoculated heavily with the sediment from the digested specimen and, after flaming, the plug is pushed into the tube to leave about 5 mm. empty space above it and warm paraffin poured in to a depth of 2 to 3 mm., allowed to harden and a second application made. After this hardens a hole is punched through to the plug, allowing an adequate gas exchange while preventing excessive dehydration of the medium. Characteristic colonies of tubercle bacilli appear after about three weeks' incubation. The liquid medium of Dubos is probably the most sensitive providing that the lipase present in the usual bovine albumin preparations is eliminated. Dubos and Davis[17] have reported that inoculums as small as two cells grow regularly. Preliminary tests with tuberculous specimens have given suggestive results.[18] Culture is not identification, of course, and acid-fast bacilli are occasionally found in non-tuberculous lesions which grow like the tubercle bacilli.[19]

[17] Dubos and Davis: Jour. Bact., 1948, 55:11.
[18] Foley: Proc. Soc. Exp. Biol. Med., 1946, 62:298; Goldie: *ibid.*, 1947, 65:210.
[19] See the discussion by Corey: Amer. Rev. Tuberc., 1945, 52:36.

Immunity. The immune response of the animal body to the presence of the tubercle bacillus is indicated by the appearance of agglutinins, precipitins, opsonins and complement-fixing antibodies in the serum. This response is not marked, however, for these antibodies are present only to low titers. None of them, with the possible exception of the complement-fixing antibodies, is of diagnostic value.[20]

The most striking immune response is the development of a hypersensitivity of the delayed type to the bacillary cell substance. Within limits, this allergic response is protective against reinfection, as shown by Koch's early experiments. He showed that the subcutaneous inoculation of the normal guinea pig with tubercle bacilli produces no immediate response but that in ten to fourteen days a nodule develops which breaks down to a persistent tuberculous ulcer and the regional lymph glands become swollen and caseous. In the tuberculous animal, however, an indurated area appears within a day or two, and there is slight necrosis with the formation of a shallow ulcer which heals promptly without the development of gross tubercle tissue or the invasion of the adjacent lymphatics by the bacilli. This resistance to reinfection is relative, of course, and is not shown unless the primary infection is of some weeks' standing or if large numbers of bacilli are injected. Animals may be sensitized, not only by infection with virulent bacilli, but also by the inoculation of attenuated or killed bacilli. Sensitization with preparations of the bacillary cell substance is difficult, however, and very large doses must be administered. Raffel[21] has shown that the delayed, non-passively transferable reaction is a result of inoculation with tubercle bacillus protein combined with a purified wax fraction consisting of an ester of polysaccharide and higher alcohols with hydroxy fatty acids; the protein alone produces an immune response with formation of precipitins.

Tuberculin.[22] The sensitized animal will react to the soluble cell substance of the tubercle bacillus, preparations of which have been called *tuberculin.* Tuberculin is usually prepared from the human type of tubercle bacillus, though bovine tuberculin is practically as active as human tuberculin in infections with the human bacillus; avian tuberculin, although considerably less active, will also produce a reaction. A variety of tuberculins have been prepared of which only a few need be noted here. The first of these was made by Koch, and consisted of the filtrate of a glycerol-broth culture of the bacilli concentrated by evaporation on a water bath to about one-tenth its original volume (the activity is heat-stable). This material is "original" or "old" tuberculin (TO or OT). A "new" tuberculin (TR—tuberculin residuum) was prepared by Koch in 1897 by macerating living virulent bacilli and extracting the mass with water, and then making an emulsion of the residuum. He later advocated the use of an emulsion (BE—bacillary emulsion or *Bazillenemulsion*) of the entire substance of young virulent bacilli in 20 per cent glycerol, actually a vaccine. Denys introduced the use of the unaltered filtrate from broth cultures (BF—broth filtrate). None of these later innovations, however, proved to be superior to old tuberculin, and the original preparation, or slight modifications of it, has been widely used.

[20] *Cf.* Klopstock: Brit. Jour. Tuberc., 1941, 35:146.
[21] Raffel: Jour. Inf. Dis., 1948, 82:267.
[22] *Cf.* the discussion by Parish: Tubercle, 1938, 19:337.

The active principle of tuberculin is protein in nature, and the cultivation of the tubercle bacillus in glycerol-asparagin-citrate synthetic solutions by Long and Seibert has made possible the study of the active principle in purified preparations. The activity is associated with a number of protein fractions, one of which Seibert prepared in crystalline form. A more satisfactory preparation of low molecular weight, *ca.* 2000, has been isolated by Seibert by precipitation with trichloracetic acid. Originally designated SOTT (synthetic medium old tuberculin trichloracetic acid precipitated), it is now known as PPD (purified protein derivative).[23]

The comparative merits of OT and PPD have been the subject of a series of investigations in recent years. OT is relatively unstable in dilutions, while PPD, a dry powder, is "dry-diluted" with lactose and is, of course, indefinitely stable in this form. Different lots of OT vary somewhat in their activity; the activity of PPD preparations is relatively constant. It appears that PPD is quite as satisfactory as OT in actual use and, because of its stability and constant activity, is regarded by many as superior to OT.

The Tuberculin Reaction.[24] Three types of reaction may be elicited in the sensitized, *i.e.*, infected, animal by the injection of tuberculin. In addition to a local inflammatory reaction at the site of inoculation, there is a focal reaction manifested as an acute congestion around tuberculous foci which, if marked, may aggravate the pathologic process, and a constitutional reaction in which the temperature rises to a peak of 102° to 104° F. and subsides in twelve to eighteen hours. In man the constitutional reaction also includes malaise, pain in the limbs and, perhaps, vomiting, dyspnea and other symptoms. These reactions do not appear in normal animals. The utility of tuberculin is, then, twofold; it may be used for diagnostic purposes and it has therapeutic value, though the latter is strictly limited.

The diagnostic tuberculin test in man is generally a skin test. Koch's original method consisted of subcutaneous injection of tuberculin. The cutaneous reaction of von Pirquet involves the rubbing of tuberculin on to the scarified skin. In the Mantoux test, the one most commonly used today, graded doses of tuberculin are injected intradermally, usually starting with 0.01 mg. of OT and going as high as 1.0 mg. or even 10 mg. on rare occasions. (0.1 ml. of a 1 : 100 dilution of OT is supposed to contain 1 mg.; the standardization of new batches is biological and carried out in guinea pigs infected with virulent tubercle bacilli.) Smaller amounts of PPD are used, since it is in dry, pure form; usually 0.00005 to 0.005 mg. A "patch test" has been introduced by Vollmer[25] in which squares (0.8 cm.) of thin filter paper, impregnated with tuberculin about four times as strong as the original old tuberculin and dried, are taped on the cleansed skin over the sternum or upper edge of the trapezius. The patch test appears to be somewhat less sensitive than the intracutaneous test of Mantoux. Since these are all skin tests only the local inflammatory reaction is observed in infected persons.

[23] See the reviews by Seibert: Amer. Rev. Tuberc., 1941, 44:1; Bact. Rev., 1941, 5:69; Chem. Rev., 1944, 34:107.

[24] See the general comprehensive discussion by Long: Amer. Rev. Tuberc., 1939, 40:607.

[25] *Cf.* Vollmer and Goldberger: Amer. Jour. Dis. Children, 1937, 54:1019; *ibid.*, 1938, 56:584; *ibid.*, 1939, 57:1272; *ibid.*, 1939, 58:527; Mandel: Arch. Pediatrics, 1945, 62:393.

In young children a positive tuberculin reaction may be taken as indicative of infection. It was formerly believed that, once established, the hypersensitivity persists essentially throughout life and that the tuberculin reaction is of limited value in the adult. It is becoming apparent, however, that reversion is more frequent than had been generally realized, particularly with reduction in the prevalence of the disease and therefore the risk of reinfection. Furthermore, there is some evidence that the correspondence of a positive tuberculin reaction and either healed or clinically significant tuberculous infection may not be as close as generally believed.[26]

The tuberculin test in cattle has great diagnostic importance, however, and has been widely used in the United States and to a lesser extent elsewhere. Three types of test may be used in cattle: the intradermal test; the ophthalmic reaction of Calmette, in which tuberculin is dropped into the conjunctiva and the reactive animal responds with the development of a diffuse congestion and edema in six to eight hours which fades away in twenty-four to thirty-six hours; and the constitutional reaction as indicated by a rise in temperature following the injection of tuberculin. The intradermal inoculation into the skin of the caudal fold is generally practiced in this country.

The therapeutic value of tuberculin may be directly observed in lupus or tuberculous infection of the skin. As indicated above, there is a reaction about the foci of infection manifested as an acute congestion and sloughing off of tissue. When it was first introduced tuberculin was regarded by many as a highly effective specific therapeutic agent for tuberculosis. Its use is, however, exceedingly dangerous, and, with the exception of lupus, the response of tuberculous infection to the injection of tuberculin has been disappointing. The therapeutic use of tuberculin has diminished greatly in recent years. It may be noted that the minute quantities used in the tuberculin test do not affect tuberculous lesions.

The Mechanism of Immunity. Infection with the tubercle bacillus confers a definite protection against reinfection, as "immunity to superinfection" resembling that observed in syphilis. The factors involved are as yet obscure. Some current opinion favors the view that the development of an allergic state is indicative of an effective immunity.[27] In this connection Brosius and Woodruff[28] have pointed out what has been casually noticed by many workers, namely that in individuals giving a positive tuberculin reaction living cells are usually free of tubercle bacilli and the bacteria are present in necrotic areas separated by an avascular barrier, while in the infected individual giving a negative reaction, tubercle bacilli are found in great numbers in living tissue. The antibodies produced are apparently not significant and antisera have no protective or curative properties. As indicated above, the development of hypersensivity is the most obvious response to infection, and there is no doubt that hypersensitivity plays a part in acquired resistance as indicated in Koch's early experiments. Its relative importance is, however, not at all clear, and the

[26] Lumsden, Dearing and Brown: Amer. Jour. Pub. Health, 1939, 29:25; Dahlstrom: Amer. Rev. Tuberc., 1940, 42:471.

[27] Lotte: Bull. Office Internat. d'Hyg. Publique, 1946, 38:1021; Negre and Bretey: *ibid.*, 1946, 38:1034.

[28] Brosius and Woodruff: Amer. Rev. Tuberc., 1944, 50:473.

mechanism of what seems to be a low-grade immunity of short duration remains at the moment largely a matter of speculation.

Active Immunization. The possibility of active immunization to tuberculosis has been of great interest since the discovery of the tubercle bacillus. In general, two types of vaccines have been employed, suspensions of living attenuated bacilli and suspensions of killed bacilli.

The attenuated bovine strain of Calmette, BCG, has been regarded as the most promising immunizing agent and has been extensively studied. It was originally given as an oral vaccine in France in the early 1920's. However, the combination of inadequate statistical data and an incident in Lübeck, Germany, in which a virulent strain was inadvertently substituted for the vaccine strain, resulting in tuberculosis in inoculated persons, put it into disrepute. Nevertheless immunization with BCG was further studied in the Scandinavian countries, beginning in 1925 in Sweden and in 1927 in Norway and Denmark. The vaccine is given intracutaneously in doses of 0.05 to 0.15 mg. and a positive tuberculin reaction appears in six to ten weeks in over 90 per cent of those inoculated. The hypersensitivity lasts for about four years, though in some persons there is reversion in as little as one year. On the assumption that a positive tuberculin reaction is indicative of immunity, reversion is taken to mean that reinoculation is required. Sufficient time has now elapsed to permit judgment as to the harmlessness and efficacy of BCG vaccine. Swedish experience is summarized by Wallgren,[29] Norwegian by Hansen,[30] and Danish by Holm,[31] and that of the Scandinavian countries as a whole by Birkhaug.[32] In addition to these, shorter term immunization studies have been carried out with nurses in Canada by Ferguson,[33] and in the United States with American Indians by Aronson and Palmer[34] and with infants by Rosenthal.[35] In general the foregoing studies have indicated that the immunization confers an appreciable, but not absolute, degree of protection against primary infection which is somewhat less than that given by the natural infection. For example, in the group studied by Aronson and Palmer the total incidence per 1000 person-years in the six year period of observation was 24.3 and 4.7 in the control and immunized groups respectively. Perhaps more important is the apparently high degree of protection against the immediate consequences of primary infection and the more severe forms of the disease.

These conclusions are not universally accepted without reservation. These recent reports have been considered critically by Wilson,[36] who is not so optimistic and emphasizes the living nature of a vaccine whose virulence is not fixed, the necessity of separation of the inoculated infant for some weeks before and after vaccination, the immaturity of the immunity mechanism at birth, etc., as deterrents to a general application of the immunization procedure in the more highly civilized countries. In the United States the reported results

[29] Wallgren: Bull. Office Internat. d'Hyg. Publique, 1946, 38:1052.
[30] Hansen: Tubercle, 1944, 25:1.
[31] Holm: Pub. Health Repts., 1946, 61:1298.
[32] Birkhaug: Amer. Rev. Tuberc., 1947, 55:234.
[33] Ferguson: Amer. Rev. Tuberc., 1946, 54:325.
[34] Aronson and Palmer: Pub. Health Repts., 1946, 61:802.
[35] Rosenthal, Leslie and Loewinsohn: Jour. Amer. Med. Assn., 1948, 136:73.
[36] Wilson: Brit. Med Jour., 1947, ii:855.

are regarded as highly suggestive of the efficacy of such immunization, but BCG vaccination is considered to be in only an experimental stage as yet.[87]

Perhaps to be regarded in the category of attenuated living bacilli is the "turtle bacillus" vaccine of Friedmann.[88] This microorganism, *Mycobacterium chelonei*, is of the "cold-blooded" variety and was isolated from a turtle. It is quite ineffective as an immunizing agent and is of only historical interest.

Suspensions of killed tubercle bacilli have been used in the immunization of cattle and experimental animals. The Spahlinger vaccine[39] is of this type. Although the resistance of guinea pigs and cattle appears to be raised by inoculation, the "immunity" so produced is of a very low order.[40] The inoculation of human beings with suspensions of killed bacilli has not been attempted on a large scale.

Fig. 148. Avian tubercle bacillus. Acid-fast stain of a smear from a pure culture. × 1050.

Pathogenicity for Lower Animals. Tuberculosis of lower animals living under natural conditions is probably very rare. Animals in captivity, however, may contract the infection with some facility; in animals kept in zoos and in monkeys in the laboratory for experimental purposes tuberculosis is not uncommon. Domestic animals may be similarly affected. A wide variety of animals may, of course, be infected experimentally with one or another of the types of tubercle bacilli.

Domestic Animals. The most commonly infected domestic animals are cattle, pigs and chickens. Cattle are infected with the bovine type of tubercle bacillus almost exclusively; they are not completely resistant to the human type, as Koch originally thought, but infection is accomplished with some difficulty. The proportion of cattle infected increases with advancing age and, in the absence of control measures, may reach 70 to 90 and possibly 100 per cent in animals

[87] *Cf.* Pub. Health Repts., 1947, 62:346.
[88] *Cf.* Friedmann and Aronson: Jour. Inf. Dis., 1929, 44:222.
[89] Spahlinger: Lancet, 1932, i:309.
[40] *Cf.* Report on the Spahlinger Experiments in Northern Ireland. H. M. Stationery Office, London. 1935.

kept in stalls. The natural infection is generally of a chronic, slowly progressive nature. The lymphatics are most frequently involved and may be the only tissues to show lesions. The lungs are also commonly affected. Lesions on the pleura have a peculiar characteristic appearance, the so-called "perlsucht" disease. The liver, spleen and kidneys are less frequently involved, but infection of the mammary glands is not uncommon. It may be noted that tubercle bacilli may be excreted in the milk in the absence of detectable lesions of the mammae. Congenital tuberculosis in cattle occurs with some frequency.

Tuberculosis in chickens is very common and is exclusively an infection with the avian variety of the tubercle bacillus. With the exception of parrots and certain birds of prey, birds are highly resistant to infection with the human and bovine varieties, and it is probable that natural infection with these types rarely if ever occurs. Tuberculosis in chickens is usually a chronic process and is characterized by the formation of nodules in the abdominal viscera. The lungs are less frequently affected.

CHARACTERISTICS OF THE VARIETIES OF TUBERCLE BACILLI

Variety	Physiology				Pathogenicity		
	Opt. temp.	Rate of growth	Pigment	Stimulation by glycerol	Guinea pig	Rabbit	Chicken
Human	37° C.	eugonic	+	+	++++	±	—
Bovine	37° C.	dysgonic	—	—	++++	++++	—
Avian	40–42° C.	rapid	+	+	+	++	++++

Pigs suffer from natural infection with both bovine and avian tubercle bacilli derived from infected cattle and poultry; they are also susceptible to infection with the human variety. In young pigs infection with bovine bacilli is generalized and acute with lesions in the lymphoid tissue, abdominal viscera and lungs. Infection with the human bacillus is generally localized, but the avian type may produce a generalized infection.

Other domestic animals suffer from tuberculosis to a considerably lesser extent. Horses, dogs and cats are occasionally infected, and the disease is rare in sheep and goats.

Experimental Animals. Experimental animals vary in their susceptibility to the varieties of the tubercle bacillus and in the type of infection produced. Of these animals only two need be considered here, the guinea pig and the rabbit. The guinea pig is highly susceptible to both bovine and human bacilli, and death follows the subcutaneous injection even of small doses in six to fifteen weeks. The lymphatic glands, spleen and liver are most affected, the lungs only slightly and the kidneys never. The necrotic areas in the spleen and liver are the most striking feature of the gross pathology and are peculiar

to the guinea pig. True tubercles are seldom seen except in the very early stages of the disease.

Rabbits are highly susceptible to infection with the bovine bacillus, somewhat so to the avian bacillus and quite resistant to the human variety. Injection of bovine bacilli produces a generalized infection that terminates fatally in two to three months. On autopsy tubercles may be found in the spleen and liver, but the lesions are most marked in the lungs and kidneys and may even be confined to them. Very large doses of human bacilli (10 to 50 mg. intraperitoneally) may produce a progressive infection but never the acute
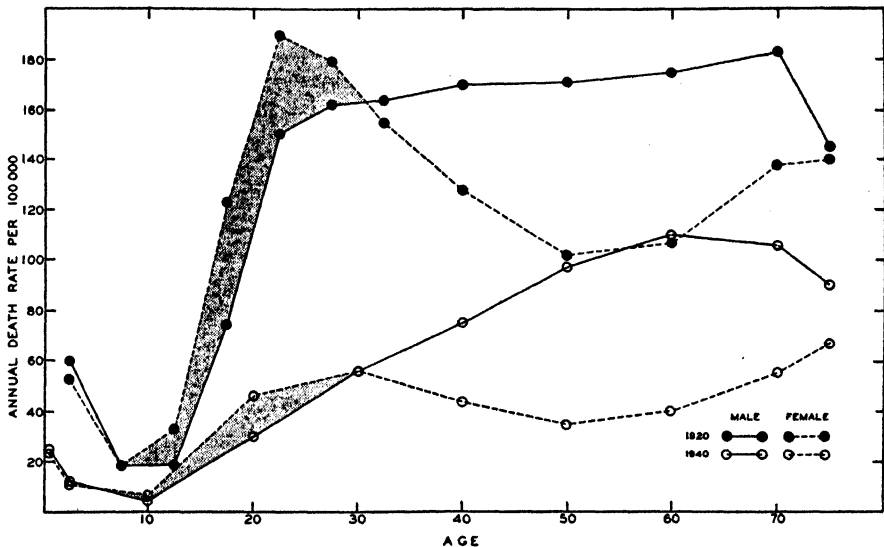
Fig. 149. The age and sex distribution of tuberculosis mortality (all forms) in 1920 and 1940. Note the differential incidence and decline over the twenty-year period. If differentiation between white and colored rates is made for 1940, the picture is almost the same, *i.e.*, the colored rates are very similar to the 1920 rates for both white and colored. Data from the reports of the Census Bureau.

fatal miliary disease. The mammalian tubercle bacilli, indistinguishable by cultural or serological methods, may, then, be sharply differentiated by their pathogenicity for these two experimental animals.

Neither rabbit or guinea pig is particularly susceptible to infection with the avian bacillus though avian tuberculosis may be produced in the rabbit.[41] In the guinea pig death may be produced by large intraperitoneal doses, but on autopsy macroscopic tubercles are not visible; cultures and smears of the liver and spleen, however, show the presence of the bacilli. This form of tuberculosis, the proliferation of the bacilli without macroscopic tubercle formation, is known as the *Yersin type* of tuberculosis.

Epidemiology. In man tuberculosis is largely an air-borne infection. Dissemination by milk is of relatively minor importance, particularly in the United States. The disease is one of civilization in that its transmission is

[41] See Feldman: *Avian Tuberculous Infection.* Williams and Wilkins Co., Baltimore. 1943.

facilitated by close contact. The frequency of positive tuberculin reactions rises rapidly from zero in the newborn to perhaps 90 per cent or over at puberty, and, as indicated earlier, almost all adults have been infected at one time or another. The infection is, then, widely disseminated through the human population. In most instances, however, clinical tuberculosis has not occurred and the lesions have healed. The proportion of persons with active clinical tuberculosis is not known; in some investigations the ratio of cases to deaths has been found to be as high as 10 : 1 or 12 : 1, but the ratio of reported cases to deaths is not much more than half of this. The prevalence of tuberculosis, then, defies precise definition.

There appear to be racial differences in susceptibility to tuberculosis. The death rate of the Negroes in this country is considerably higher than that of the white population, though the incidence of clinical tuberculosis in the two races is not greatly different. Whether this higher death rate is a consequence of environmental conditions or whether it is in part attributable to racial differences in susceptibility has been a point of considerable interest. It is probable that both factors are involved and that there is a real racial difference in susceptibility. In this connection the experience in the United States Army is of particular interest. Roth[42] has found that in the years 1922–36 the average white morbidity rate was 2.10 per 1000 and the Negro rate 2.56, a ratio of 4 : 5; the white death rate was 0.24 and that of the Negroes 0.99, a ratio of 1 : 4; and the case-death ratios were 8.75 for the whites and 2.61 for the Negroes. Under the controlled conditions prevailing, *i.e.*, preliminary physical examination, age and sex selection, the same housing conditions and identical diagnostic and therapeutic facilities, it would appear to be definitely established that though the incidence of clinical tuberculosis is no greater in the Negro, the disease is much more frequently fatal and the indicated greater susceptibility is a consequence of racial rather than environmental factors. Aronson[43] has reported similar findings; during World War II the Negro, while making up but 10 per cent of the United States Army, contributed 43.4 per cent of the total deaths from tuberculosis. There is some evidence that less well-defined races of man differ in their resistance to tuberculosis; Jews and Italians appear to be more resistant than the Irish.

The age and sex distribution of mortality from tuberculosis must be considered in relation to the decline in the disease rather than as isolated phenomena. That tuberculosis has been decreasing at a steady and relatively rapid rate since 1850 or thereabouts is indicated by the decline in the death rate from this disease. As shown in Fig. 150, the death rate from all forms of tuberculosis in the registration area of the United States has declined from 190.5 per 100,-000 in 1900 to 40.1 in 1945, and a decline of this general order is apparent elsewhere. As in the case of some other diseases, the decline set in before the discovery of the bacterial etiology of infectious disease and the development of preventive measures, and hence is by no means entirely attributable to the practice of preventive medicine.

This decline has not been relatively the same in either the various age groups or in the two sexes. The death rate is highest in the very low age groups, that

[42] Roth: Amer. Rev. Tuberc., 1938, 38:197.
[43] Aronson: Milit. Surgeon, 1946, 99:491.

of one to two years, and falls rapidly in the five to nine group and then rises to a peak in early adult life, twenty to twenty-four, and declines with, in recent years, a small secondary peak between forty-five and fifty-four years (late adult tuberculosis). This secondary peak is a relatively recent development and is regarded by Frost[44] as a residual of higher rates in earlier life rather than a postponement of maximum risk. The decline in tuberculosis in the present century has been relatively greatest in the very young, an undoubted consequence of preventive measures.

The sex distribution of the death rates from tuberculosis in the various age groups is a curious phenomenon which has not been explained. The death rate for males of all ages is somewhat higher than that for females. In young adults, *i.e.*, the fifteen to twenty-nine age group, the female death rate is considerably higher than the male death rate. In the higher age groups the male
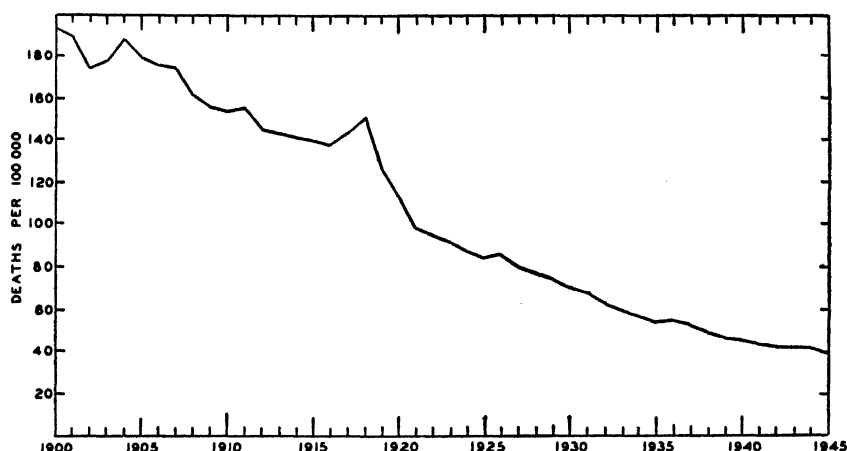
Fig. 150. The prevalence of tuberculosis, all forms, in the Registration Area since 1900 as indicated by the uncorrected death rates. From data compiled by the Bureau of the Census.

death rate becomes proportionately greater and exceeds the female rate for the rest of life. In the present century the female death rate in the higher age groups has declined somewhat more rapidly than the male death rate. The present status of the relation between the sex-specific death rates may be illustrated by the ratio of male to female deaths in the United States registration area in 1931–34 as given by Dauer.[45] For all ages the ratio of male to female deaths was 1.24, at ages under ten the ratio was 1.22, at ten to thirty years 0.69, at thirty to fifty-five years 1.64, and at fifty-five and over 1.57. (See Fig. 149.)

Tuberculosis mortality has fallen at a more accelerated rate than deaths from all causes; in 1900 more than 11 per cent of all deaths were due to tuberculosis, in 1940 the percentage had fallen to 4.3. At the beginning of the century tuberculosis was first in numerical importance as a cause of death and it was seventh in 1940. Although the reported cases and deaths have de-

[44] Frost: Amer. Jour. Hyg., Sec. A, 1939, 30:91.
[45] Dauer: Amer. Rev. Tuberc., 1938, 37:435.

clined markedly, there has not been any appreciable alteration in the proportion of cases to deaths, *i.e.*, the case fatality rate.[46]

It will be clear that the observed decline in tuberculosis is a complex matter for which there is no simple explanation. Although unknown factors are undoubtedly involved, it appears highly probable that the environmental improvements implied in the more decent living of the last few decades have contributed in no small part to the decrease in tuberculosis. The early detection and isolation of cases practiced in recent years has very likely had its effect also. Frost[47] has pointed out that the observed decline is indicative of the inability of the disease to reproduce itself; a surplus of chances to establish infection is, of course, essential and the required level of transmission may be very high and necessitate not only universal but frequently repeated exposure to relatively large doses of bacilli. If the requisite level is not maintained the disease must necessarily die out. In certain other diseases the causative agent may be disseminated by healthy persons; in tuberculosis, however, it is only the "open" clinical case that discharges bacilli into the adjacent environment. The present practice of isolation of active cases would appear, then, to be a sound one, and it is perhaps not unreasonable to hope for the eventual eradication of this disease.

THE BACILLUS OF LEPROSY (MYCOBACTERIUM LEPRAE)[48]

Like tuberculosis, leprosy is an old disease of man. Perhaps more prevalent in ancient times, it is now most common in Central Africa, India, Japan and other Asiatic countries, and it was estimated that there were about 3,000,000 lepers in the world in 1924. The disease is prevalent in South America, with endemic centers in Brazil, Colombia and Argentina, and an overall incidence of more than 1 per 1000 population. Practically all the Caribbean islands are infected to about the same extent. It is relatively uncommon in Europe and sporadic cases occur in Latvia, Estonia, southern and eastern Russia, and on the Mediterranean coast.

Leprosy has been introduced into the United States with varying consequences. In Louisiana, Florida and Texas the imported cases have established foci in which the disease has a tendency to perpetuate itself, while in California and the central northwestern States it tends to die out. Elsewhere in the country transmission is so rare as to be negligible.[49] It is estimated that there are 500 to 1000 lepers in this country, many of whom are segregated in the National Leprosarium in Carville, Louisiana, the remainder living for the most part in California and New York.

Bacilli were found by Hansen in 1872 in the round epithelioid cells generally known as lepra cells, and the observation was one of the first of pathogenic bacteria.

Morphology and Staining. Morphologically the leprosy bacilli closely resemble the tubercle bacilli. They are long (6 μ), slender rods, usually straight, but sometimes slightly curved. They are non-motile and do not pro-

[46] *Cf.* Drolet: Amer. Rev. Tuberc., 1938, 37:125.

[47] Frost: Amer. Rev. Tuberc., 1935, 32:644.

[48] For a detailed consideration see Rogers and Muir: *Leprosy.* Williams & Wilkins Company, Baltimore. 1940.

[49] McCoy: Pub. Health Repts., 1942, 57:51.

duce spores. They generally occur within the cells but are sometimes found free in the lymph spaces. Their arrangement within the cells is characteristic, several bacilli being usually grouped together in bundles like packets of cigarettes.

The staining reaction of these microorganisms is much like that of the tubercle bacilli. They stain somewhat more readily than the latter, and also decolorize more quickly with acids, but the difference is not great enough for differentiation. The presence of large numbers of bacilli within the cells, together with the clinical features of the disease, makes it possible to distinguish leprosy bacilli from tubercle bacilli without difficulty. Because of their acid-fast staining characteristic and their morphological resemblance to the tubercle bacilli and similar bacteria, these bacilli are included with the mycobacteria and designated *Mycobacterium leprae*.

Fig. 151. The leprosy bacillus. Acid-fast stained smear from a skin lesion. Note the characteristic tendency to parallel arrangement of the bacilli in packets. × 1800.

Cultivation. Numerous unsuccessful attempts to cultivate Hansen's bacillus on artificial media were made for years by bacteriologists all over the world. A few investigators have reported positive results. In most instances acid-fast bacilli have been cultivated, but in others a variety of microorganisms, including diphtheroids, actinomycetes and anaerobic bacilli, have been found. It has been suggested by some workers that the acid-fast bacilli present in leprous lesions represent but a single stage in a developmental cycle and outside the body other forms appear. Kedrowski[50] cultivated a highly pleomorphic microorganism with branched, acid-fast and non-acid-fast stages. Clegg[51] also obtained apparent growth and continued multiplication in subculture on an artificial medium of an acid-fast bacillus. His results were confirmed by others, in particular Duval,[52] who reported cultivating a bacterium regarded as *Myco. leprae* directly from human tissue on artificial media. Acid-fast bacilli, some

[50] Kedrowski: Ztschr. f. Hyg., 1901, 37:52.
[51] Clegg: Philippine Jour. Sci., 1909, 4:403.
[52] Duval: Jour. Exp. Med., 1910, 12:649.

of them chromogenic, have been cultivated by other workers, and there are at present a number of cultures in various laboratories labeled "*Myco. leprae.*"

It seems highly probable that not any of these bacteria are leprosy bacilli in that they are etiologically related to the disease in man.[53] They are very likely best grouped with the saprophytic acid-fast forms such as the smegma and timothy bacilli.

The cultivation of the bacilli of leprosy has been intensively investigated in recent years by Soule and McKinley, who, in 1932, first isolated in culture a very slow-growing, non-chromogenic acid-fast bacillus which closely resembled the bacilli observed in leprous nodules. On media suitable for the growth of tubercle and other acid-fast bacilli incubated in an atmosphere of 40 per cent oxygen and 10 per cent carbon dioxide for six weeks, tiny, discrete colonies appeared. Although a number of isolations failed to grow on subculture, two strains have been maintained through 40 transfers over

RELATIVE PROPORTION OF TYPES OF LEPROSY*

Region	Neural Type	Lepromatous Type
Africa	90.5%	9.5%
Philippines	50.0%	50.0%
Mexico	40.0%	60.0%
Java	29.0%	71.0%

* Data from Lowe: Proc. Sixth Pacific Sci. Congr., 1942, *5*:921.

a period of six years.[54] These cultures were obtained from leprous material in Puerto Rico. Two years later Soule[55] reported that of 42 specimens obtained in the Philippines, 25 yielded cultures of the same or a very similar bacterium, and of these two strains were maintained on repeated subculture. The intradermal inoculation of rhesus and Cebus monkeys resulted in the production of temporary granulomatous lesions which could not be transmitted in series. Whether these bacilli are, in fact, leprosy bacilli is not clear; proof demands the reproduction of the disease, but since inoculation of experimental animals with relatively large amounts of macerated leprous nodules has not produced the disease, the negative results with cultures are, perhaps, not significant. More recently Loving[56] has reported the cultivation of the bacilli in a thiamine-enriched medium.

Pathogenicity. Although any organ or tissue in man may be attacked with varying results, two distinct types of leprosy are usually recognized—the nodular and the anesthetic. The former, which is the more acute, is char-

[53] Four strains of "leprosy bacilli" isolated in recent years were intensively studied at the National Institute of Health, but there was no evidence of a causal relation to the disease. *Cf.* Nat. Inst. Health Bull. No. 173, 1940.

[54] Soule and McKinley: Amer. Jour. Trop. Med., 1932, *12*:1, 441; Jour. Amer. Med. Assn., 1932, *98*:361; Amer. Assn. Advncmt. Sci., Symposium Ser., 1938, *1*:87; McKinley: Int. Jour. Lepr., 1939, *7*:1,217.

[55] Soule: Proc. Soc. Exp. Biol. Med., 1934, *31*:1197.

[56] Loving: Amer. Jour. Trop. Med., 1943, 23:593.

acterized by the development of masses of granulation tissue, the so-called "leproma," which may appear superficially in different parts of the body, and by their growth and coalescence produce distortion and mutilation. The anesthetic type, or nerve leprosy, progresses more slowly, the average duration of the cases being nearly twice as long (eighteen years) as cases of the nodular type, some being known to extend over thirty-five or forty years. Atrophy of the muscles and other trophic disturbances accompany the nerve lesions. Very many lepers die of other diseases; Kean and Childress,[57] for example, reported that, of a group of 82 lepers autopsied, 24 died of tuberculosis, 22 of neuritis, 15 of leprosy, 10 of heart disease, 4 of cancer, and the remainder of miscellaneous diseases other than leprosy.
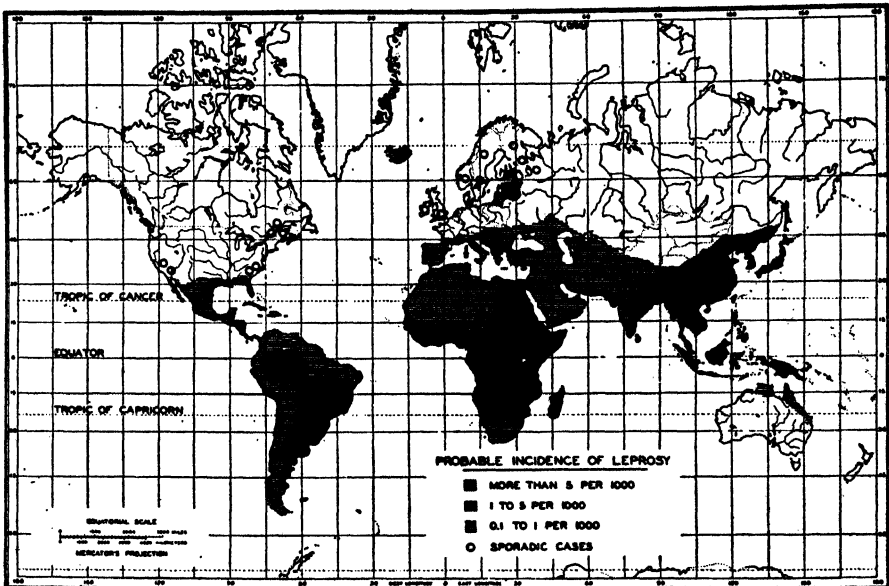
Fig. 152. The probable prevalence of leprosy in the world as indicated by present data. Based on Goode *Base Map* No. 201 M. By permission of the University of Chicago Press. (After Saunders.)

In both forms of leprosy Hansen's bacillus is found in all cases, in enormous numbers as a rule in the lesions of nodular leprosy, and less abundantly in the anesthetic type. Very few bacilli are observed outside the body cells, and they are found in the cytoplasm and do not invade the nucleus. Almost any part of the body may be the site of leprous growth; the kidneys are usually invaded, the liver and spleen always. The bacilli have been seen in the central nervous system and are occasionally found in the blood, generally in the leucocytes but sometimes free.

Leprosy has never been produced in experimental animals by the inoculation of leprous material from man; even the implantation of nodules containing enormous numbers of bacilli has been without effect.

[57] Kean and Childress: Internat. Jour. Leprosy, 1942, 10:51.

Transmission. Leprosy is probably transmitted by contact, though the conditions that make transmission possible are entirely unknown. There are numerous instances in which healthy persons, such as asylum attendants, have been more or less in contact with lepers for long periods without contracting the disease. In other cases, however, leprosy has been contracted by those in close and long-continued contact with diseased individuals. There is also indirect evidence of transmission. Manson[58] cites the case of an Irishman who acquired leprosy in the West Indies. On his return to Ireland his bed was shared by his brother who, moreover, sometimes wore the leper's clothes. The brother, who had never been in any foreign country, became in time an undoubted leper. In this instance communication from one person to another was practically demonstrated. Currie[59] found that a large percentage of cases studied in Hawaii gave a history of exposure, and that usually such exposure was of an intimate character. Saunders[60] has pointed out that leprosy occurs for the most part in populations of low social order living under unsanitary conditions of poverty, crowding, uncleanliness and sickness. Even so a relatively small proportion of those exposed develop the disease. Doull et al.[61] have shown that age at the time of exposure is important, the risk being greatest for those exposed before five years of age. The average interval between exposure and development of leprosy in their study was 10.5 years for those exposed under ten years of age, and 6 years for those exposed over ten years of age.

One way in which the bacillus may leave the body is in the nasal mucus, and large numbers of the bacilli are found in the secretions of the nose in many cases. Bacilli may sometimes be discharged from the mouth or nose in small particles of mucus. In the opinion of many writers, the mucous membrane of the nasopharynx is the point at which the bacteria are introduced into the body, as well as the chief source from which infection is spread.

Evidence of the direct inoculability of leprosy from man to man is quite inadequate. Many attempts to infect healthy persons have been made and have failed, and one often-cited instance of successful inoculation is by no means unimpeachable. In the case of the criminal Keanu in the Hawaiian Islands, reported by Arning,[62] implantation of material from a leprosy nodule was followed by the development of true leprosy, which terminated fatally six years after inoculation. The experiment, however, did not include the important source of error involved in the facts that Keanu was a native of a country in which leprosy was common, that he had lived among lepers, and that members of his family were lepers. More recently Lazoudaky[63] inoculated himself intramuscularly with blood from two lepers and contracted the disease.

Much light is thrown on the contagious character of leprosy by the success that has attended the isolation and segregation of leprous patients. The Norwegian experience showed that a careful but not unduly rigorous system of

[58] Manson: *Tropical Medicine*. London. 1900, p. 448.
[59] Currie: Pub. Health Bull. No. 41, 1910.
[60] Saunders: Proc. Sixth Pacific Sci. Congr., 1942, 5:957.
[61] Doull, Guinto, Rodrigues and Bancroft: Amer. Jour. Trop. Med., 1945, 25:435.
[62] Arning: Arch. f. path. Anat., 1893, 134:319.
[63] Lazoudaky: Jour. Trop. Med. Hyg., 1937, 40:77.

separation was accompanied by a diminution of the number of cases from 2870 in 1856 to 577 in 1900. The circumstance that infection does not invariably follow chance contact or association should not, therefore, lead to neglect of the facts that leprosy is a bacterial disease; that up to the present under natural conditions the specific bacterium has not been found except in the human body; and that, so far as is definitely known, the leper himself is the most important means by which leprosy spreads.

Immunity. Man is clearly highly resistant to infection with the leprosy bacillus, and, after infection is established, the ensuing disease is essentially a benign process that may take many years to terminate fatally; in fact, many lepers die from other causes. Little is known regarding the specific immune response. Lepers do, however, develop a hypersensitivity to the cell substance of acid-fast bacilli such as the tubercle bacillus and the various saprophytic species. A curious fact is the appearance of a positive Wassermann reaction in lepers in the absence of syphilis. According to some, this is an artifact arising from infection with yaws, and it is said that the Kahn test is negative in non-syphilitic lepers. Recent investigations,[64] however, have shown that both the Wassermann and the Kahn tests may be positive in lepers having no clinical symptoms of syphilis.

Lepromin. The hypersensitivity developed by the leper may be demonstrated by the intradermal inoculation of material prepared from leprous nodules. Two reactions are observed, one early and occurring after three or four days, and the late reaction appearing three to four weeks after inoculation. The reaction is not specific in that a response is also elicited by preparations of other acid-fast bacilli. Dharmendra[65] has isolated a nucleo-protein from leprosy nodule material, however, which appears to have some specificity. In its present form, at least, the lepromin test has little or no diagnostic utility.

Rat Leprosy. A native disease of wild rats, commonly known as rat leprosy, and characterized by enormous numbers of acid-fast bacilli present in the lesions, was described by Stefansky in 1903, as occurring in wild rats in Odessa. The disease was observed in the same year by Dean, who later showed it to be transmissible. It has since been observed in wild rats all over the world.[66]

The acid-fast bacilli closely resemble the leprosy bacillus in size and shape and are found intracellularly but not as often in the packet arrangement of parallel bacilli. *Myco. leprae murium* has not been cultivated on artificial media. The disease may, however, be transmitted to white rats, mice and guinea pigs by inoculation with pieces of tissue.[67] The experimental infection is a relatively benign process. A local, circumscribed lesion develops following subcutaneous inoculation which becomes palpable in four to five weeks and eventually develops to a large tumorous mass ulcerating on the surface and

[64] Badger, Patrick, Fite and Wolfe in Nat. Inst. Health Bull. No. 173, 1940; Faget and Ross: Ven. Dis. Information, 1944, 25:133.

[65] Reviewed by Dharmendra and Lowe: Leprosy Rev., 1946, 17:9.

[66] See the review by Lowe: Int. Jour. Lepr., 1937, 5:311, 463; also Fielding: Med. Jour. Australia, 1945, 32:473.

[67] For recent studies on experimental rat leprosy see Fite in Nat. Inst. Health Bull. No. 173, 1940.

persisting throughout the life of the rat. The earliest lesions in the other organs do not appear before four to six months, and the animal dies only after a year or more.

The relation of rat leprosy to human leprosy is not known. Rats are not susceptible to inoculation with human leprous material.

OTHER ACID-FAST BACILLI

Mycobacterium Paratuberculosis.[68] A chronic enteritis of cattle usually terminating fatally is caused by an acid-fast bacillus closely resembling the avian variety of the tubercle bacillus. The disease is sometimes called *Johne's disease* and the bacillus *Johne's bacillus* after its discoverer. The disease only remotely resembles tuberculous infection. The lesions in the intestinal wall are proliferative and the granulomatous tissue may contain epithelioid cells and occasionally giant cells, but there is no caseation.

The disease appears to be widespread in the United States. Infected cattle become hypersensitive to the bacillary substance and filtrates of cultures produce a skin reaction analogous to the tuberculin reaction which is designated the "johnin reaction." No case of human infection with *Myco. paratuberculosis* has been recorded.

The Vole Bacillus. An acid-fast bacillus responsible for an epizootic, chronic infection of the field vole, *Microtus agrestis*, resembling tuberculosis was discovered by Wells[69] in 1937. It closely resembles the tubercle bacillus culturally though it forms no pigment and growth is not enhanced by glycerol. It is pathogenic for both guinea pigs and rabbits, considerably more so for the latter, and is not pathogenic for fowls. Brooke[70] has suggested that it is a distinct type of mammalian tubercle bacillus and should be called *Mycobacterium tuberculosis* var. *muris*. This microorganism has been of particular interest because, though it produces only a localized and retrogressive infection when inoculated in small doses in guinea pigs and calves, tuberculin sensitivity is produced, and preliminary experiments on its use as a prophylactic have given suggestive results.

The "Cold-Blooded" Mycobacteria. Acid-fast bacili have been found associated with pathologic processes in various cold-blooded animals. In some instances the processes superficially resemble tuberculous lesions. *Myco. piscium* was isolated from nodules and tumor-like formations in carp; *Myco. marinum* from "tuberculosis" of sea bass and certain other salt-water fish; *Myco. ranae* was found in the liver of a frog; *Myco. thamnopheos* is a parasite in garter snakes; and *Myco. chelonei*, the "turtle bacillus," has been referred to above.

The saprophytic acid-fast bacilli include the well-known timothy bacillus, *Myco. phlei*, found in soil, on grasses and elsewhere in nature; the "butter bacillus," *Myco. butyricum*; and *Myco. smegmatis*, which is, however, a parasite found in both male and female smegma. The smegma bacillus is often difficult to distinguish from the tubercle bacillus on morphological grounds, and confusion of the two may have considerable practical importance

[68] Hagen and Thomson: Tr. Nat. Tuberc. Assn., 1931, p. 232.
[69] See the general review by Wells: Med. Res. Council Spec. Rept. Ser. No. 259, 1946.
[70] Brooke: Amer. Rev. Tuberc., 1941, 43:806.

in the diagnosis of suspected cases of tuberculous infection of the urinary tract. It is, it may be noted, also found in the urine and may contaminate fecal specimens. The saprophytic bacilli all grow much more rapidly than the tubercle bacilli, and neither they nor the bacilli isolated from cold-blooded animals are pathogenic for guinea pigs and rabbits, or at best only feebly so. The interrelationships of these non-pathogenic acid-fast bacilli are considered at length by Gordon[71] and Gordon and Hagan.[72]

[71] Gordon: Jour. Bact., 1937, 34:617.
[72] Gordon and Hagan: Jour. Bact., 1938, 36:39.

MEDICAL MYCOLOGY: THE PATHOGENIC ACTINOMYCETES, MOLDS, YEASTS AND RELATED MICROORGANISMS[1]

It has been pointed out in preceding chapters that the tubercle bacilli and the diphtheria bacilli are more closely related to the fungi than are the "true bacteria." These forms may be regarded as connecting links between the bacteria and a number of the fungi proper, some of which are pathogenic for man and lower animals. The discovery of the causal relation of certain of the fungi to infectious disease preceded the pioneer work of Pasteur and Koch with the pathogenic bacteria by several years, for Schoenlein studied the fungus causing favus in 1839, and in the same year Lagenbeck described the yeast-like microorganism of thrush. In spite of its earlier beginnings, medical mycology was soon overshadowed by bacteriology and has never received as much attention though some of the fungous diseases are among the more common infections of man. This is perhaps attributable to the relatively benign nature of the common mycoses and the rarity of the more serious ones, and to the morphological basis of the differentiation of these structurally complex forms which, in a practical sense, sets them off sharply from the bacteria.

Even a brief consideration of the fungous diseases makes it clear that they fall into two well-defined groups, the superficial mycoses and the deep-seated mycoses.[2] The superficial mycoses are by far the most common, are caused for the most part by a relatively homogeneous group of fungi, the *dermatophytes,* and include the various forms of tinea or ringworm with infection of the hair and hair follicles, the superficial infections of the intertriginous or flat areas of the glabrous skin, and the onychomycoses or fungous infections of the toe and fingernails. In general, the lesions are mild, superficial and restricted; the infections are almost never fatal. The causative microorganisms are parasitic

---

[1] For a general discussion of medical mycology see: Skinner, Emmons and Tsuchiya: *Henrici's Molds, Yeasts and Actinomycetes.* 2d ed. John Wiley & Sons, New York. 1947. A detailed consideration of the pathogenic fungi with little or no critical evaluation of their relative importance is given by Dodge: *Medical Mycology.* C. V. Mosby Company, St. Louis. 1935. An excellent, though largely clinical, discussion of the dermatophytes is presented by Lewis and Hopper: *Introduction to Medical Mycology.* Year Book Publishers, Chicago. 1939. A very brief discussion of the general subject is given by Swartz: *Elements of Medical Mycology.* Grune & Stratton, New York. 1943. Clinical and diagnostic aspects of the mycoses are discussed in detail in Conant *et al.: Manual of Clinical Mycology.* W. B. Saunders Company, Philadelphia. 1944. Nickerson *et al.: Biology of Pathogenic Fungi.* Chronica Botanica, Waltham, Massachusetts. 1947.

[2] The general characteristics of the fungous diseases are discussed by Henrici: Jour. Bact., 1940, 39:113.

and the infection is transmitted from one host to another. The deep-seated mycoses, on the other hand, are relatively rare and are heterogeneous in etiology; they include actinomycosis, aspergillosis, sporotrichosis, blastomycosis, coccidioidomycosis and cryptococcus or torula infections. The causative micro-organisms appear to be free-living parasites for the most part and are usually not transmitted from one individual to another, but the lesions they produce are severe, deep and spreading, and a fatal outcome is not uncommon.

THE FUNGOUS INFECTIONS

Type	Causative Organism	Disease
Super-ficial infections	Dermatophytes: *Microsporum, Trichophyton, Achorion, Epidermophyton, Endodermophyton*	Ringworm of scalp and glabrous skin, suppurative folliculitis, onychomycosis
	Actinomyces minutissimus	Erythrasma
	Erysipelothrix rhusiopathiae	Erysipeloid in man
	Fonsecaea pedrosoi and its varieties	Chromoblastomycosis
	Candida albicans and related forms	Moniliasis of the mucous membranes and nails, sometimes generalized
Deep-seated infections	*Actinomyces bovis, A. asteroides, A. madurae,* etc.	Actinomycosis, including actinomycotic mycetoma
	Actinomyces muris-ratti	Rat-bite fever
	Aspergillus fumigatus, and others	Apergillosis, otomycosis and pulmonary mycosis
	Sporotrichum schenckii	Sporotrichosis
	Debaryomyces neoformans	European blastomycosis, torula meningitis
	Blastomyces dermatitidis	American blastomycosis
	Coccidioides immitis	Coccidioidomycosis
	Histoplasma capsulatum	Histoplasmosis

The criterion of pathogenicity is one of the poorest that can be used in the differentiation of microorganisms, not only because it is variable and difficult to determine, but because by its use parasitic microorganisms are grouped together that are, in fact, much more closely related to certain free-living forms than they are to one another. The superficial nature of pathogenicity as a differential characteristic is nowhere better illustrated than among the fungi, for the pathogenic forms which constitute the subject matter of medical mycology form a heterogeneous group and include some of the actinomycetes, certain molds and mold-like fungi, and a number of yeasts and yeast-like

organisms. The pathogenic varieties of these fungi are an almost infinitesimally small portion of existing fungi, the vast majority being saprophytic forms found in the soil and elsewhere playing a role of primary importance in the cyclical transformations of organic matter, especially in the decomposition of hemicelluloses and lignins. A number are of great industrial importance, as in cheese manufacture, saccharification of starches, etc. From a general biological point of view, then, the pathogenicity of certain fungi is of very minor significance; from that of the parasitized host, man, it is of considerably greater interest.

The fungi are structurally complex, showing a variety of reproductive structures associated with sexual and asexual processes, in addition to vegetative non-reproductive elements. Their differentiation into genera, species and varieties is made in large part on a morphological basis, especially the morphology of the reproductive structures, and, in contrast to the bacteria, their physiological and immunological characteristics are usually of minor or no importance for purposes of differentiation or identification. The biochemistry of the fungi, and the molds in particular, has been extensively investigated, beginning with the work of Wehmer and more recently by Raistrick and his colleagues[3] in connection with the elucidation of decompositions occurring in nature and their application to industrial processes. Present knowledge of the respiratory mechanism of the cell (p. 63) and of alcoholic fermentation (p. 92) derives in very large part from studies of yeasts. The immunological properties of the fungi have been little studied aside from noting the immunological relation of certain of the actinomycetes to the tubercle bacilli, and the allergic phenomena associated with infection with certain of the dermatophytes and yeast-like fungi.

Though a great many species of fungi have been described as pathogenic for man and animals, not all are of equal importance. Some, especially among the dermatophytes, are not legitimate species different from those already known. Furthermore, in many cases the fungus described probably had no causal relation to the pathologic process from which it was isolated and in others only one or two cases of infection have ever been observed. It is the accumulation of large numbers of species of fungi and the minutiae of their morphological differentiation that give medical mycology its complexity. Here we shall be concerned only with the more important fungi known to be causally related to human disease; the remainder, though large in number, are associated with only a small fraction of one per cent of fungous disease of man.

I. THE ACTINOMYCETES

The actinomycetes occupy an uncertain position with respect to the bacteria on the one hand and the fungi on the other and are regarded by many as transition forms. The order Actinomycetales is made up of two families, the Mycobacteriaceae which includes the mycobacteria and the corynebacteria, and the Actinomycetaceae which includes Actinomyces, Erysipelothrix and

---

[3] *Cf.* Raistrick *et al: Studies in the Biochemistry of Microorganisms*, Phil. Trans. Roy. Soc., 1931, Ser. B, 220:1–367. Later papers will be found for the most part in the Biochemical Journal.

Leptotrichia; the last is made up entirely of saprophytic forms and does not concern us here.

The actinomycetes grow in the form of fine straight or wavy non-septate filaments or hyphae 0.5 to 0.8 μ in diameter which show both lateral and dichotomous branching, and which may grow out from the medium to form an aerial mycelium. On solid media the filaments occur in tangled masses while in liquid media there is a tendency to centers or clumps of growth. Small oval to rod-shaped spores 1 to 2 μ long are formed by aggregation of the protoplasm in some of the hyphae, spore formation progressing from the tip. The maturation of the spore-bearing hyphae is often associated with the formation of spirals which range from open, barely perceptible coils to those which are so compressed that adjacent turns are in contact. The spirals may be dextrorse or sinistrorse and both direction and tightness of coiling are constant within
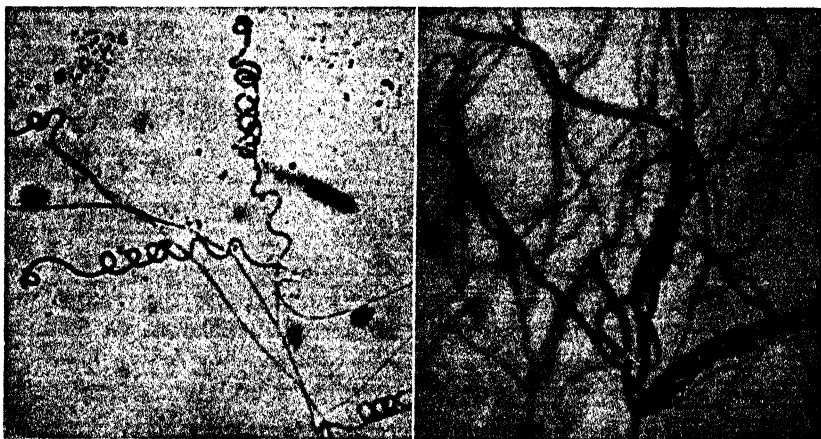
Fig. 153. Actinomycetes. Left, showing branched filaments with coiled terminal bodies which are fragmented into bacteria-like cells. Right, *A. antibioticus* showing both submerged mycelium and aerial sporulating mycelium. $\times$ 1200 (Starkey, Waksman and Woodruff).

species. The spores are more resistant than the filaments and will survive 60° C. for as long as three hours, but are less resistant than bacterial spores. The tips of other filaments may become swollen and club-shaped. Segmentation of the filaments occurs, in some species as early as twenty-four hours while in others it is delayed three weeks or more; the segmented filaments break up to form bacillary bodies 4 to 6 μ in length which are morphologically indistinguishable from many true bacteria. In most smear preparations of the pathogenic forms, the filaments are broken up and the appearance is that of ordinary bacilli. All, or practically all, of the actinomycetes are gram-positive and some of the pathogenic forms are acid-fast; these last are immunologically related to the mycobacteria.

Both spores and fragments of mycelium grow in subculture. On solid media the growth is dry, tough and leathery, sometimes wrinkled, adherent to and piled above the medium; in many instances it resembles that of the myco-

bacteria. In some cases the growth appears powdery or chalky owing to the formation of aerial mycelium. In liquid media growth occurs in the form of a dry, wrinkled surface film or, more often, as flakes or aggregates which adhere to the sides of the flask, especially at the surface, or sink to the bottom.

Pigment formation, with colors ranging over the entire spectrum, is common among the actinomycetes and differentiation is usually made between pigmentation of the vegetative mycelium and the spore-bearing aerial mycelium as well as pigment diffusing into the medium. Soluble purple and brown pigments are often observed on protein-containing media. The actinomycetes, especially the saprophytic forms, are physiologically active, utilizing a variety of carbon and nitrogen compounds, and many are actively proteolytic. An earthy to musty odor like that of freshly turned soil is produced by many species. The optimum temperature for growth is usually 20° to 30° C. though some of the pathogenic species grow at 37° C. and thermophilic species, analogous to thermophilic bacteria, are known. The great majority of actinomycetes are aerobic, but some of the pathogenic forms are anaerobic or at least must be cultivated under reduced oxygen tension.

Differentiation of the actinomycetes from one another is in part on a morphological and in part on a physiological basis, the latter including pigmentation and proteolytic activity. It has become increasingly apparent that there are two relatively well-defined morphological groups. The one, which includes all the pathogenic forms, is characterized by failure to form aerial mycelium and a marked tendency of the hyphae to segment and fragment into bacillary forms, often very early in the development of the culture. The other group is much larger, contains only saprophytic forms, and is characterized by the production of spores in aerial hyphae. There has been some tendency to separate the two by different generic names. The name Nocardia has been widely used, both for the entire group of actinomycetes and for the saprophytic forms only. Other names such as Discomyces, Streptothrix, Cladothrix, etc., have been proposed and used to a minor extent but are generally regarded as invalid for one reason or another. The name Proactinomyces was introduced by Jensen[4] in 1931 for the pathogenic actinomycetes and has gained some currency in recent years though it has not been generally accepted. The question has recently been considered by Waksman and Henrici[5] who separate the two groups by making them different families, the family Actinomycetaceae being retained for the pathogenic forms and including two genera, Actinomyces for the anaerobic forms, and Nocardia for the aerobic forms. A new family, Streptomycetaceae, is created for the saprophytes; questions of nomenclature and relationships of these forms need not be considered here.

The name Actinomyces will be used here for the pathogenic actinomycetes, all of which are characterized morphologically by failure to form aerial mycelium or spores. This group may be further subdivided on the basis of oxygen requirements and acid-fast staining properties. In addition, a number of more or less diverse microorganisms are grouped with the actinomycetes by some workers. The so-called pleuropneumonia organisms (p. 547) are so considered

[4] Jensen: Proc. Linnean Soc. N. S. Wales, 1931, 57:364.
[5] Waksman and Henrici: Jour. Bact., 1943, 46:337.

by Ledingham[6] and certain of the non-sporulating obligate anaerobes (p. 540) are sometimes regarded as actinomycetes. Other microorganisms of uncertain position but showing affinities for the actinomycetes, including Erysipelothrix, the rat-bite fever fungus, and actinobacillus are discussed in this section.

THE PATHOGENIC ACTINOMYCETES AND ACTINOMYCETE-LIKE FUNGI

Organism	Disease	Geographic Distribution
Actinomyces bovis	Actinomycosis in man, "lumpy jaw" in cattle	Ubiquitous; majority of cases reported in U. S.
Actinomyces madurae *Actinomyces bahiensis* *Actinomyces somaliensis*	Actinomycotic mycetoma in man	Tropics, including India and Egypt; few cases in U. S.
Actinomyces asteroides *Actinomyces gypsoides*	Deep-seated abscesses and pulmonary infection	Ubiquitous
Actinomyces keratolyticus	"Cracked heels"	India
Actinomyces minutissimus	Erythrasma	Ubiquitous
Actinomyces farcinicus *Actinomyces dermatonomus*	Animal parasites, do not infect man	Europe and Australia respectively
Actinomyces muris-ratti	Rat-bite, Haverhill fever	Europe and U. S., probably ubiquitous
Actinobacillus lignieresi	Actinobacillosis in cattle, very rare in man	South America, not in U. S.
Erysipelothrix rhusiopathiae	Natural parasite of hogs, erysipeloid in man	Europe and U. S., probably ubiquitous

ACTINOMYCES BOVIS

Though the term actinomycosis refers to any infection with an actinomycete, it is often thought of in connection with disease caused by *Actinomyces bovis*. This is the most clearly defined and best studied actinomycete infection and the disease occurs chiefly in cattle, and in few other animals; it is also occasionally seen in man. Although the disease was undoubtedly observed early in the nineteenth century, actinomycotic tumors being described by Leblanc[7] in 1826 under the name of osteosarcoma, it was first recognized as a specific parasitic disease by Bollinger[8] in 1877. At his instigation the fungus was studied by the botanist Harz[9] who described it and named it Actinomyces or ray fungus, because of the ray-like structure of its growth in the tissues, but did not cultivate it. In 1891 Wolff and Israel[10] isolated a microaerophilic actino-

[6] Ledingham: Jour. Path. & Bact., 1933, 37:393.
[7] Leblanc: Jour. Méd. Veterin., 1826, p. 333.
[8] Bollinger: Deut. Ztschr. f. Tiermed., 1877, 3:334.
[9] Harz: Deut. Ztschr. f. Tiermed., Suppl., 1878, 4:125.
[10] Wolff and Israel: Virchows Arch. f. path. Anat., 1891, 126:11.

mycete from pathologic material by anaerobic culture and in the same year an aerobic actinomycete was isolated by Bostroem[11] from similar sources and named *Actinomyces hominis* (sometimes called *Actinomyces graminis*). Bostroem's organism has been proved to have been a contaminant, however, and it is definitely established that the organism isolated by Wolff and Israel (called by some *Actinomyces israeli*) was that observed by Bollinger and Harz and the etiologic agent of the disease. These early observations were extended in large part through the work of Wright[12] and Emmons[13] and present knowledge has been summarized by Erikson.[14]

Morphology and Staining. *Morphology in the Tissues.* Actinomycosis is essentially a suppurative process, characterized by the formation of granula-
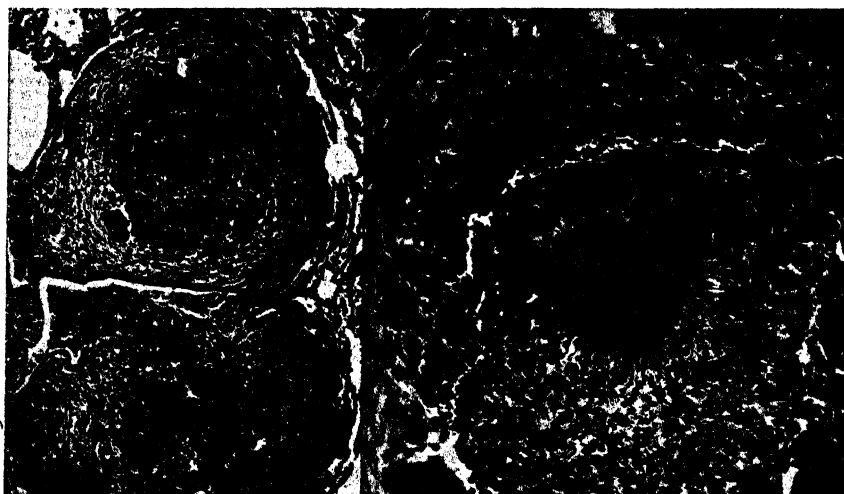
Fig. 154. Actinomycotic (*A. bovis*) granule in rabbit lung. Hematoxylin and eosin. Left, two granules; × 80. Right, upper part of left field at higher magnification; × 300. Note the eosinophilic clubs at the periphery of the granule (Rosebury, Epps and Clark).

tion tissue, fistulation, sinus formation and by the presence in the pus of yellow granules ("sulfur granules"), the *Drüsen* of German writers. These are in fact colonies of the fungus and when examined microscopically are seen to consist of dense rosettes of club-shaped filaments in radial arrangement. The individual rosettes are usually 30 to 40 μ in diameter but sometimes are as large as 200 μ. The minute yellow granules, visible to the naked eye, may consist of a single rosette or may be made up of several. The rosette itself is made up of three kinds of structures: a central core of branching filaments, irregularly disposed but with a general radial arrangement; refringent, club-shaped bodies at the periphery radially arranged; and spherical coccus-like bodies. The granules may

---

[11] Bostroem: Beitr. Path. Anat. Allg. Path., 1891, 9:1.

[12] Wright: Pub. Mass. Gen. Hosp., Boston, 1905; Jour. Med. Res., 1904-5, 8:349.

[13] Emmons: Puerto Rico Jour. Pub. Health & Trop. Med., 1935, 11:63; Pub, Health Repts., 1938, 53:1967.

[14] Erikson: *Pathogenic Anaerobic Organisms of the Actinomyces Group.* Med. Res. Council (Great Britain) Spec. Rept. Ser. No. 240, 1940.

be crushed and examined in fresh preparations in which the clubs may be plainly seen or a stain such as eosin may be used which colors the sheath of the clubs. The filaments are gram-positive and the stain is useful for tissue sections; hematoxylin-eosin is quite satisfactory for sections. *A. bovis* is not acid-fast.

The filaments of the central core are branched, are often curved, sometimes spirally, and are thickly interlaced in a network of mycelium. The individual filaments have a granular appearance and, particularly in older granules, segmentation and fragmentation are common, giving the filaments the appearance of chains of cocci.

The club-shaped bodies at the margin of the granule are conspicuous for their high refringency and general structureless, homogeneous appearance. They are pear-shaped swellings of the terminal ends of the filaments, and arise

Fig. 155. *A. bovis* from cultures. Left, darkfield × 900. Middle and right, gram stains of rough and smooth cultures respectively. × 1200 (Rosebury, Epps and Clark).

as distinct tranformations of these. In young colonies the hyaline substance of which the clubs are composed is soft and may be dissolved in water, but as the age of the colony increases, the clubs become of firmer consistency. Their formation appears to be associated with the resistance of the tissues; when resistance to invasion is slight, they are absent, filaments alone being found. Clubs are, as a rule, more common in bovine than in human lesions.

The coccus-like bodies reported by various observers are probably of diverse nature. Such forms may result from the segmentation and fragmentation of filaments; in other cases they may be the ends of clubs appearing in the field of focus of the microscope. In still others they may perhaps be micrococci, secondary invaders of the suppurating actinomycotic lesion. (See p. 672.)

Morphology in Culture. The colonial morphology of *A. bovis* grown anaerobically on solid media is sufficiently distinctive that, with experience, the colonies are readily differentiated from those of contaminating bacteria.[15] After four to six days' incubation the colonies are often less than 1 mm. in diameter. They are usually opaque, dead white or rarely showing a slight gray

[15] *Cf.* Rosebury, Epps and Clark: Jour. Inf. Dis., 1944, 74:131.

or yellow tinge, and have a pitted glistening surface and irregular margin. The growth, piled high above the surface of the medium, is similar to that of the tubercle bacillus. The colonies are tough and leathery, often coming away from the medium in one piece, and are difficult to emulsify. It has been observed that after continued cultivation the colonies tend to become smooth and less distinctive in appearance, and may be much softer in consistency. In shake cultures in dextrose agar the fungus grows below a depth of perhaps 10 mm. and appears as irregularly shaped, opaque, whitish nodules which may reach a diameter of 2 to 3 mm. in a week. Stab cultures in dextrose agar give a dense grayish streak of small colonies along the lower part of the line of inoculation. In broth good growth usually occurs in the form of solid, whitish, mulberry-like granules at the bottom of the tube; the broth does not, as a rule, become cloudy.
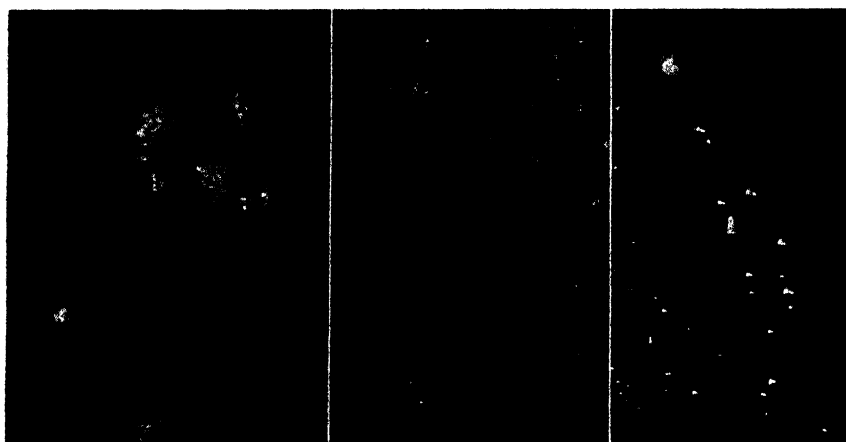
Fig. 156. Colonies of *A. bovis* on brain heart infusion agar after six days' incubation. Left and center are rough types; × 3. The colonies on the right are the smooth type; × 6 (Rosebury, Epps and Clark).

The essential features of the rosette have been reproduced in cultures. The smaller colonies are rounded masses of branching and interlacing filaments. As the filaments become older they tend to fragment and the largest colonies are dense opaque masses of short filaments and rod forms. Clubs are not formed in the usual media but only in the presence of blood, serum or ascitic fluid and even there develop inconsistently. Stained smears of cultures show largely bacillary forms and a few fragments of branching filaments. The bacillary forms may be diphtheroid in appearance, stain irregularly, and some have terminal swellings; the last are quite unlike the peripheral clubs of the actinomycete granule.

Physiology. Particular interest has centered about the relationship of *A. bovis* to oxygen. In the past it has been assumed that this microorganism is an obligate anaerobe and the identity of strains showing even scanty growth under aerobic condition has been questioned. It now seems to be established that it is microaerophilic with a limited oxygen tolerance, and some strains may grow sparsely in the air for one or two transfers. It is, however, best cultivated

under anaerobic conditions in the presence of 5 to 10 per cent carbon dioxide. The limits in *p*H are similar to those of most bacteria and growth is best at 37° C. Growth is relatively slow even under optimum conditions and four to six days' incubation are usually required.

There appears to be no definite information regarding the nutritive requirements of the fungus; in general it does not appear to be fastidious in that reasonably good growth occurs in meat extract media with added dextrose (1 per cent). Rosebury, Epps and Clark[15] have found brain heart infusion agar most satisfactory and not improved by the addition of blood. Growth on potato is scanty and milk is not a good culture medium. It has been a general experience that *A. bovis* cultures are difficult to maintain in the laboratory and usually die out in spite of weekly or biweekly transfer. Erikson[14] has recommended alternate transfers on different media for the maintenance of cultures and Rosebury, Epps and Clark[15] report good results with this method. Glucose, maltose, lactose and salicin are fermented with acid and no gas, indol and hydrogen sulfide are not produced, and nitrate is actively reduced. These biochemical reactions have no differential significance.

Pathogenicity for Animals. The majority of actinomycotic lesions in cattle are found in or about the head; the lower jaw and tongue in particular are affected, hence the common names of "lumpy jaw" and "wooden tongue" for this disease. In addition to the growth in the tongue and maxillary bones, actinomycotic lesions occur in the pharynx, lungs, skin, lymph glands and subcutaneous tissue, especially of the head and neck, and occasionally in other organs, notably the udder and liver. The growth of the parasite usually leads to the formation of a hard tumor which gradually increases in size and burrows into the adjacent tissues, softening and disintegrating the bony structure of the head, and at the same time forming new tissue, so that great distortion often ensues. The extension of the disease takes place by gradual invasion of the contiguous tissues, metastases being uncommon. When death occurs it is not, as a rule, due to any toxic effect but wholly to the mechanical action of the tumor in pressing upon or occluding the respiratory passages or in interfering with the taking or mastication of food. Generalized actinomycosis is rare. When it occurs the bloodstream rather than the lymph seems to be the channel by which the disease is spread. Secondary abscesses are found mainly in the liver.

The characteristic yellow granules or *"Drüsen"* are found in the suppurating mass of the tumor; if the pus is shaken up with water in a test tube the small granules become evident and sink to the bottom of the tube. These structures are so typical that their demonstration by microscopic examination in a case of obscure suppuration is sufficient to establish a diagnosis. The new growth—granulation tissue—consists chiefly of epithelioid and spindle-shaped connective-tissue cells; small giant cells are sometimes present. To the naked eye, actinomycotic lesions in the lung and udder often resemble tuberculous nodules and at times have undoubtedly been mistaken for them; microscopic examination suffices to establish their nature.

The incidence of actinomycosis in cattle is uncertain, in part because it is readily confused with actinobacillosis; possible something under 1 per cent is a fair estimate. Of the other domestic animals swine are most frequently affected,

usually with primary lesions in the mammary glands. Horses and sheep are affected less often and the disease seldom occurs in dogs and is rare in cats.[16]

Experimental Animals. Attempts to infect experimental animals with *A. bovis* have in general been disappointing in that only a small proportion of inoculated animals develop the disease and the lesions are limited and benign. Henrici and his associates[17] found that repeated inoculations are much more effective; Slack[18] was able to produce chronic progressive actinomycosis in this way in four of five rabbits. Rosebury, Epps and Clark[15] were less successful, and by repeated inoculation of twenty-four rabbits and sixteen guinea pigs produced infection in only five animals, progressive and fatal in two guinea pigs and one rabbit and localized and benign in two rabbits. In the experimental disease, however, the essential features of the natural infection are observed, including the formation of tubercle-like nodules and the development of structurally typical granules with clubs.

Pathogenicity for Man. Infection with *A. bovis* is observed in man from time to time, but it is not common. Sanford[19] found about 700 reported cases in the United States up to 1923; a large proportion were in the upper Mississippi Valley, a distribution which probably represents recognition rather than incidence of the disease.

The disease in man differs in minor respects from that in cattle. Actinomycotic infections of the bone are relatively less frequent, the disease being confined to the softer parts in most cases. There is usually a lesser production of new tissue and a more extensive softening and suppuration. The disease falls into three clinical types. About 60 per cent of the infections are *cervico-facial* and this type is often associated with dental defects or accidents and is a chronic, localized form of the disease which is relatively benign and usually susceptible to treatment. Some 14 per cent of the cases are *thoracic* infections and 8 to 18 per cent are *abdominal* in which the primary lesion is often in the appendix; in these types prognosis is poor.[20] Draining sinuses are usually found in all types and abscesses are frequently observed in the liver at autopsy. Generalization by hematogenous spread is occasionally seen and is relatively more common in man than in cattle. Meningitis and endocarditis have been reported. The characteristic granules with clubs are usually found in the pus but may be absent occasionally in draining sinuses, especially when the infection has spread rapidly, particularly in meningitis and empyema. The disease may terminate fatally in a few weeks through secondary infection or formation of emboli, or it may drag along in a chronic form for many years; spontaneous healing has been observed.

Epidemiology. Direct transmission of *A. bovis* from one animal to another or from cattle to man has never been satisfactorily established. Many of the cases of actinomycosis reported in man are among persons who have not been engaged in agricultural pursuits and, so far as discovered, have not come in contact with any preexisting cases in animals or man. Nor has *A. bovis* been

---

[16] Martin: Univ. Pennsylvania Vet. Ext. Quart., 1942, 87:15.

[17] Mathieson, Harrison, Hammond and Henrici: Amer. Jour. Hyg., 1935, 21:405.

[18] Slack: Jour. Bact., 1942, 43:193.

[19] Sanford: Jour. Amer. Med. Assn., 1923, 81:655.

[20] *Cf.* Sanford and Voelker: Arch. Surg., 1925, 11:809. Good: Arch. Surg., 1930, 21:786.

found to exist as a saprophyte in nature. There has been some confusion on this point since Bostroem's aerobic actinomycete is a widely distributed saprophyte; the Wolff-Israel organism, however, appears to be a strict parasite and as such is widely distributed in the normal human mouth and throat. Actinomycetes make up a large part of dental plaque material (p. 537) and fungi indistinguishable from *A. bovis* and capable of producing experimental actinomycosis in animals have been found in the absence of actinomycosis in carious teeth, dental and gingival scrapings and tonsillar crypts by many workers.[21] In fact it was early predicted by Wright[12] that *A. bovis* would be found to be a normal inhabitant of the mouth. In the light of present knowledge it seems highly probable that the infection is endogenous in origin and that the fungus, normally present but of low pathogenicity, multiplies in the favorable environment
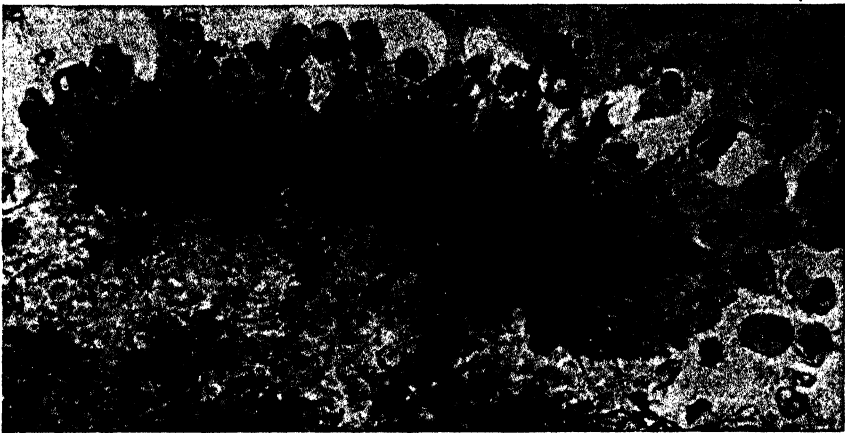
Fig. 157. Pulmonary infection in man with *Actinomyces bovis*. Margin of a granule in lung tissue showing the ray-like structure. Note the bacteria designated *Actinobacillus actinomycetem-comitans* in the center of the granule. Gram stain; × 1000 (Humphreys).

provided by necrotic tissue resulting from injury, and occasionally establishes a focus of infection, possibly in association with bacteria which are eliminated as the infection progresses.

Immunity. There appears to be little effective acquired immunity to *A. bovis* infection. Recently the possibility of the development of a hypersensitivity to the actinomycete cell substance has received some attention. The apparently greater efficacy of repeated small doses over a single massive inoculation in the experimental production of the disease has provided the basis for Henrici's[2, 17] suggestion that infection may depend on previous sensitization. This is not established as yet.

Isolation and Diagnosis. As indicated earlier, the demonstration of the typical actinomycete rosette in the tissue or pus of a specimen is sufficient to establish a diagnosis of actinomycosis in man. Actinomycosis in cattle may be confused with actinobacillosis; the two are readily differentiated by examination of a gram-stained smear for the presence of gram-positive diphtheroid-like

[21] *Cf.* Lord and Trevett: Jour. Inf. Dis., 1936, 58:115. Emmons: Puerto Rico Jour. Pub. Health & Trop. Med., 1935, *11*:63. Also Slack: *loc. cit.* and Rosebury *et al.*: *loc. cit.*

fragments of actinomyces filaments, or of gram-negative actinobacilli. As may be inferred from the foregoing discussion, animal inoculation is useless as a diagnostic method. Cultivation of the fungus is often, though not always, successful.

The procedure recommended by Wright[12] for the isolation of *A. bovis* in culture has been widely used. Granules, preferably from a closed lesion, are thoroughly washed in several changes of sterile water or broth, and then crushed between sterile glass slides. The material is inoculated into liquid (40° C.) 1 per cent dextrose agar in test tubes filled to a depth of 6 to 8 cm. After incubation at 37° C. for four to eight days, characteristic colonies develop, in greatest numbers in a narrow zone 5 to 12 mm. below the surface. These may be isolated from the shake culture in the usual way, washed in sterile water or broth if there is reason to suspect bacterial contamination and subcultured in deep tubes of dextose agar. Even in the absence of contamination the method is not particularly satisfactory and frequently fails.

Rosebury, Epps and Clark[15] have shown that abundant and characteristic growth occurs on the surface of enriched agar media containing dextrose and incubated under anaerobic conditions in the presence of carbon dioxide. They recommend brain heart infusion containing 2 per cent agar. The granule, exudate, or other material is streaked serially on plates of the medium by means of a sterile bent glass rod and the plates incubated four to six days under anaerobic conditions in the presence of 5 per cent carbon dioxide. By this method *A. bovis* can be isolated even in the presence of heavy bacterial contamination.

MYCETOMA

Mycetoma (fungous tumor) or Madura foot is an affection occurring with some frequency in Southern India; the first cases were reported (1842 *et seq.*) from Madura, hence the name. It is a disease of tropical climates though a number of cases have been observed about the Mediterranean in North Africa, in Greece and Italy. It also occurs in South America and the Caribbean islands. It has been observed in temperate climates, a few cases having been reported from Europe and the United States. It was first observed in this country by Wright[22] in Boston in 1898 and according to Gammel[23] twenty-four cases had been found in this country up to 1927. The disease was first studied systematically by Vandyke Carter and the earlier work is discussed at length by Chalmers and Archibald[24] who proposed the system of classification of the mycetomas which is generally used.

The disease usually affects the foot; occasionally the hand is attacked, and other parts of the body more rarely. It commonly occurs in those habitually going barefoot and the fungus presumably enters the body through some injury or abrasion. There is some evidence that it may remain latent for some time, even a period of years, and become active following injury. The part first involved, usually the sole of the foot, shows a small subcutaneous swelling which slowly enlarges and softens to become phlegmonous. It ruptures to the

[22] Wright: Jour. Exp. Med., 1898, 3:422.
[23] Gammel: Arch. Dermat. and Syph., 1927, 15:241.
[24] Chalmers and Archibald: Ann. Trop. Med. & Parasit., 1916, 10:169.

surface, sinus tracts form and the process burrows into the deeper tissues, producing swelling and distortion of the foot; the bones may or may not be extensively involved. Numerous small eminences are found on the surface, each the orifice of a sinus. The discharge is a viscid, slightly purulent, often foul-smelling fluid containing minute granular particles up to 1 mm. in diameter. The presence of these grains separates the mycetomas from pseudomycetomatous conditions observed in yaws, sporotrichosis, etc. The disease is strictly local, secondary abscesses seldom if ever occur and the internal organs are probably never affected. Iodide therapy is said to be effective in the actinomycete infections, but with extreme tissue destruction or other etiologic agents, surgical removal of the infected tissues, usually involving amputation, is necessary and curative.

The most obvious clinical difference in the mycetomas is the color of the grains present in the discharge. These may be white, yellow or black, and red grains have been observed. These grains, or colonies, are composed of mycelial

Fig. 158. *Actinomyces asteroides.* Smear from a pure culture. Gram stain; × 1050.

filaments of the fungus; clubs are often present in the actinomycete grains, and chlamydospores in those of other fungi; and the color is due to the formation of pigment by the fungus. Differentiation into white or yellow grained types on the one hand, and black on the other, is clinically attractive but unsound since grains of the same color may be produced by widely different fungi, and in one instance both black and white grained types have been produced experimentally with the same strain of microorganism. It is customary, therefore, to divide the mycetomas into two etiologic types: —

(1) Actinomycosis—mycetoma of actinomycete etiology.

(2) Maduromycosis—mycetoma caused by molds and mold-like organisms.

It would seem that since none of the reported actinomycotic mycetomas in which the fungus was identified showed black grains, and black grains are associated with maduromycosis, the white or yellow grained type may be either actinomycosis or maduromycosis while the black grained type is probably maduromycosis. There is in general a greater tendency to bone involvement in

the actinomycete infections. Otherwise there appear to be no clinical differ-
ences between the two types. The majority of reported cases are maduromy-
cosis, but in this country eighteen of the twenty-four cases discussed by Gam-
mel[23] were actinomycosis.

Actinomycotic Mycetoma. A number of species of actinomycetes have
been described in mycetoma; Gammel[23] lists thirteen and more recently Dodge[1]
gives fifteen. Of these two appear to be the most important, the others having
been observed only occasionally. *Actinomyces madurae* is widely distributed
and has been found in Tunis, Egypt, Algiers and Greece, in Senegal, Somali-
land and Abyssinia, in India and in South America and the Caribbean. *Actino-
myces asteroides* was found in mycetoma in the Philippines and has since
been observed in South America and in Europe. Of the others *Actinomyces
bahiensis* has been found several times in Khartoum and *Actinomyces soma-
liensis* in Africa. It is of interest that *A. bovis* has been found in actinomycotic
mycetoma in South America.

Maduromycosis. The etiology of maduromycosis is much more hetero-
geneous, the causative organisms including molds and mold-like fungi which
more properly belong in the next section. Of the molds, species of Aspergillus
and Penicillium have occasionally been found in maduromycosis. The other
etiologic agents include organisms of the genus Madurella, secreting a black
pigment and producing a black grain mycetoma; there are eight species of
which the first to be described, *Madurella mycetomi,* is the most common.
Closely related forms producing no pigment and causing white grain mycetoma
are three species of the genus Indiella; these are not common. One of the
sporotrichia, Glenospora (Aleurisma), is responsible for some of the black
grain and white grain mycetomas and certain species of Monosporium (Scedo-
sporium) cause both black and white grain mycetomas.

Diagnosis. As indicated above, the mycetomas are characterized by the
presence of granules in the discharge. The grains may be very hard and are
usually treated with strong (20 per cent) sodium hydroxide to dissolve pig-
ment and debris and examined in a wet, unstained condition for the presence
of tangled mycelial filaments. Clubs may be observed in the actinomycete
infections and thallospores, heavy-walled homogeneous structures, are found
in Madurella and other infections. For differentiation and identification of the
fungus, culture is necessary, on enriched dextrose agar in the case of actino-
mycetes and on Sabouraud's medium in the case of others.

<div align="center">OTHER ACTINOMYCETES</div>

Scattered cases of various types of infection with actinomycetes are oc-
sionally reported. *Actinomyces asteroides,* originally found in a brain abscess
by Eppinger[25] in 1890, has been observed in a variety of pathologic processes
including pulmonary affections sometimes called "pseudo-tuberculosis." Its
occasional occurrence in mycetoma has been noted above. Both this organism
and *Actinomyces gypsoides,* another strain of *A. asteroides,* isolated by Hen-
rici and Gardner[26] from sputum in pulmonary disease, are acid-fast. These
forms are immunologically related to the mycobacteria, the tubercle and leprosy

[25] Eppinger: Ziegler's Beitr. z. path. Anat., 1890, 9:287.
[26] Henrici and Gardner: Jour. Inf. Dis., 1921, 28:232.

bacilli.[27] A number of acid-fast actinomycetes have been isolated from similar affections and given various names; Henrici and Gardner[26] found twenty-six such cases in the literature to 1921. Infection occurs first in the peribronchial nodes, followed by the development of a caseous bronchopneumonia with central softening and cavitation. The disease is of about six months' duration and may be confused with tuberculosis; in acid-fast stains of sputum smears only the bacillary forms of the actinomycete are present and cannot be distinguished from tubercle bacilli. It may be readily differentiated by culture and guinea pig inoculation.

A disease of the foot occurring in India, cracked heels or *keratodermia plantare sulcatum,* and known locally as *chaluni, haja,* or *panki,* has been shown by Acton and McGuire[28] to be caused by an actinomycete, *Actinomyces kera-*
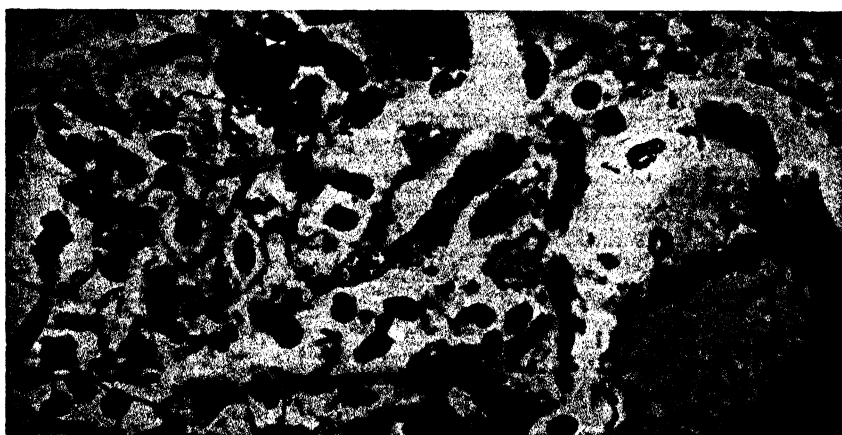
Fig. 159. Bronchopulmonary actinomycosis in man resulting from infection with the non-ray type (aerobic) of actinomyces. Note the branching mycelium in the giant cells. Gram stain; × 860 (Humphreys).

tolyticus. The fungus appears to have a lytic action on the horny layer of the epidermis and the disease is characterized by solution of the thick horny skin of the plantar surface in grooves. The skin cracks along these grooves to form deep fissures on the heel and in the thick sodden skin between the toes which extend through the corium to the subcutaneous tissues. Secondary infection, especially with streptococci, is common.

The disease *ulcus interdigitale* appears to be caused at times by the same organism. Deep fissures develop between the toes and large chronic ulcers appear which show little discharge and are very painful. The lesions may extend deeply into the interdigital cleft and secondary infection may necessitate amputation of the toe. The ulcers may also develop in the sole of the foot in the region of the heel and great toe. Infection with *A. keratolyticus* occurs during the monsoon season and appears to be contracted through walking barefoot on damp soil contaminated with manure.

Erythrasma, a superficial mycosis resembling tinea versicolor (p. 705), is

[27] *Cf.* Kedrowsky: Philippine Jour. Sci., 1937, 62:439.
[28] Acton and McGuire: Indian Med. Gaz., 1931, 66:65.

caused by *Actinomyces minutissimus* (*Microsporon minutissimum*). The initial lesion is a small scaly macule which slowly enlarges to produce a yellowish to reddish brown scaly patch with reddened borders. The disease is usually confined to the groins. The axillae, the intergluteal cleft or other intertriginous areas are less frequently involved. It is never found on free (non-intertriginous) surfaces.

Other actinomycete infections take a variety of forms. Abscesses in various parts of the body, including the brain, are observed from time to time and a number of cases of corneal infection and conjunctivitis have been reported.

Actinomycotic infections of domestic animals are occasionally observed. One of the first to be reported was a farcy-like disease of cattle caused by *Actinomyces farcinicus*. Other infections are seen in sheep and goats in a variety of clinical forms. A skin disease of sheep of some economic importance because of the copious exudate which prevents shearing and makes the wool unmarketable, and due to *Actinomyces dermatonomus*, has been reported from Australia.[29]

When actinomycetes are isolated from some pathologic process and studied, it is often found that the fungus does not conform precisely to previously described species, and a new species is created. Many species have been described, therefore, of which a large proportion are of doubtful importance either because of the rarity of their occurrence, as having been observed only once, or because there was some doubt as to the etiologic relation of the fungus to the disease. Dodge[1] lists 140 species of actinomycetes of proved or suspected pathogenicity, including 29 which he regards as inadequately described.

ACTINOMYCES MURIS-RATTI
(RAT-BITE FEVER, HAVERHILL FEVER)

A microorganism variously known as *Streptothrix muris-ratti*, *Haverhillia multiformis*, *Streptobacillus moniliformis*, *Actinomyces muris* and *Actinomyces muris-ratti* is the cause of an acute febrile disease sometimes given the descriptive name erythema multiforme. It is also called rat-bite fever because it may be acquired through the bite of infected rats.

For a good many years the etiology of the disease known as rat-bite fever was confused, for Schottmüller,[30] Blake[31] and others isolated from the blood of patients a fungus to which the name *Streptothrix muris-ratti* was given, while Japanese work indicated that a similar disease, known in Japan as *sodoku*, is caused by a spirochete. In 1925 an epidemic, milk-borne disease with unusual and distinctive clinical features occurred in Haverhill, Massachusetts, and was given the descriptive name *erythema arthriticum epidemicum* or Haverhill fever by Place, Sutton and Willner.[32] The causative microorganism was isolated by Parker and Hudson,[33] it proved to be a member of the Actinomycetaceae and was given the provisional name *Haverhillia multiformis*. In the same year Levaditi[34] independently studied a similar organism from a case clinically like Haverhill fever and named it *Streptobacillus monili-*

[29] Bull: Australian Jour. Exp. Biol. & Med. Sci., 1929, 6:301.
[30] Schottmüller: Derm. Wschr., 1914, 58: suppl. p. 77.
[31] Blake: Jour. Exp. Med., 1916, 23:39.
[32] Place, Sutton and Willner: Boston Med. & Surg. Jour., 1926, 194:285.
[33] Parker and Hudson: Amer. Jour. Path., 1926, 2:357.
[34] Levaditi: Presse Med., 1926, 34:340.

formis. Other work has yielded similar results, and it is highly probable that the fungus isolated and studied by all these workers is the same, an actinomycete for which the proper name is *Actinomyces muris-ratti.* The Japanese work has also been amply confirmed and it is now clear that there are two types of rat-bite fever, completely different in etiology. The actinomycete infection concerns us here; the spirochetal disease is considered elsewhere (p. 752).

The microorganism is, like the other actinomycetes, pleomorphic in culture with branching filaments, fragmented, bacillary and coccobacillary forms being found in stained smears. Staining may be irregular, and swollen and club-shaped cells are found. It has been suggested that this organism is related to the organisms of the pleuropneumonia group. (See p. 547.) It is not acid-fast and, in contrast to most actinomycetes, is gram-negative. It requires enriched media for growth; Parker and Hudson[33] found whole rabbit blood, allowed to clot and then inactivated, and broth containing serum or ascitic fluid excellent liquid media, and glycerin extract of potato mixed with infusion broth, enriched with egg yolk and coagulated by inspissation, the best solid medium. Löffler's serum medium is not particularly satisfactory. Growth is markedly better under anaerobic conditions in the presence of added carbon dioxide than it is in the presence of air.

When the disease in man is contracted through a rat bite, the initial wound heals but after a week or ten days becomes inflamed and painful. Adenitis develops and is followed by toxic symptoms which are the first evidence of the disease acquired from milk. These include headache, chills, vomiting and general malaise. An eruption of morbilliform character appears, especially on the extremities, and there is a multiple arthritis, often severe. The clinical resemblance to rat-bite fever of spirochetal etiology is very close, though Allbritten, Sheely and Jeffers[35] believe the two can be differentiated. The causative microorganism may be isolated by blood culture and has been found in the fluid of affected joints. Agglutinins are formed and according to Brown and Nunemaker[36] are of considerable diagnostic utility.

A. muris-ratti has been found to be a normal parasite of rats and mice and is present in the nasopharynx. It may assume pathogenicity for the rodent host and produce sporadic or epidemic disease of either an acute septicemic or chronic polyarthritic type.[37] An epizootic in wild mice of the latter type has been reported from Australia.[38] It has also been found in bronchopneumonia of rats.[39] It is clear that man may acquire the disease by the bite of normal rats and probably almost all sporadic cases arise in this way. Brown and Nunemaker[36] are of the opinion that rat bite produces the actinomycete infection much more commonly than the spirochete infection in this country. Farrell, Lordi and Vogel[40] report the fourteenth sporadic case of Haverhill fever on record, *i.e.,* in the thirteen years following its differentiation in 1926, and

[35] Allbritten, Sheely and Jeffers: Jour. Amer. Med. Assn., 1940, *114*:2360.
[36] Brown and Nunemaker: Bull. Johns Hopkins Hosp., 1942, *70*:201.
[37] Levaditi, Selbie and Schoen: Ann. Inst. Pasteur, 1932, *48*:308. See also Mackie, van Rooyen and Gilroy: Brit. Jour. Exp. Path., 1933, *14*:132; and Strangeways: Jour. Path. Bact., 1933, 37:45.
[38] Williams: Med. Jour. Australia, 1941, *1*:357.
[39] Tunnicliff: Jour. Inf. Dis., 1916, *19*:767.
[40] Farrell, Lordi and Vogel: Arch. Int. Med., 1939, 64:1.

Brown and Nunemaker[86] have reported three additional cases; the number of undiagnosed cases and the proportion of previously reported cases of rat-bite fever that were *A. muris-ratti* infections are not known (*cf.* p. 752). As indicated above, the Haverhill epidemic was milk-borne; another milk-borne outbreak involving eighty-six persons was reported[41] in 1934. Parker and Hudson[33] found evidence which suggested that the Haverhill milk was contaminated by an infected cow but this could not be proven; others have suggested contamination of milk by rats as a possibility.

ACTINOBACILLUS

Actinobacillus Lignieresi. In 1902 Ligniers and Spitz[42] isolated a non-motile, non-branching, non-acid-fast, gram-negative bacillus from the lesions of a disease of cattle which closely resembles actinomycosis and with which it is frequently confused. The microorganism is known as *Actinobacillus lignieresi* and the disease as *actinobacillosis*. Originally found in Argentina, it appears to be relatively common and has been recognized in Europe and in this country.[43]

Granules, very similar to but smaller and more numerous than the "sulfur granules" of actinomycosis, are found in the thick pus from the lesions. These granules or colonies contain clubs radially arranged about a center which is composed of detritus and gram-negative bacilli. The microorganism is pleomorphic in culture; diplococci and slender rods are found in smears from cultures in liquid media while long curved forms are present in deep colonies in agar media. The bacilli are 0.4 μ in diameter and 1 to 15 μ in length.

Surface colonies on laboratory media are small, 0.5 to 1 mm. in diameter, smooth, glistening, convex, bluish white and delicate in appearance. In liquid media, such as serum-glucose-broth, the growth consists of small grayish granules adhering to the sides of the tube and readily broken loose by shaking; broth does not become turbid. The microorganism appears to be a strict parasite and grows very poorly or not at all except in media containing serum or whole blood. It is an obligate aerobe though primary cultures are often more successful in fluid media or serum-glucose-agar stab cultures, especially when incubated in an atmosphere containing 10 per cent carbon dioxide. Cultures grow up in twenty-four hours at 37° C.; growth is very slight at 20° C. Dextrose, lactose, sucrose, maltose, raffinose and mannitol are fermented; the fermentation of xylose is irregular. Arabinose, dulcitol, salicin and inulin are not fermented. Very small amounts of indol are produced, coagulated serum is not liquefied, and litmus milk is usually unchanged but sometimes slightly acid.[44] The systematic position of Actinobacillus is uncertain. Thompson[45] has grouped it with the glanders bacillus and Bergey (1948) placed it in the family Parvobacteriaceae, with Malleomyces and Pasteurella. It is generally regarded, however, as more closely related to the actinomycetes.

[41] Place and Sutton: Arch. Int. Med., 1934, 54:659.
[42] Ligniers and Spitz: Bull. Soc. Cent. Med. Vet., 1902, 20:487, 546.
[43] *Cf.* Vawter: Cornell Vet., 1933, 23:126.
[44] There is some discrepancy in the literature on these points, including primary isolation culture, suitability of coagulated serum media, etc. Here the writer has followed Hagan: *The Infectious Diseases of Domestic Animals.* Comstock Publishing Co., Ithaca. 1943.
[45] Thompson: Jour. Bact., 1933, 26:221.

As indicated above, the disease in cattle closely resembles actinomycosis, differing in that the bones are seldom affected and the lesions are found in the soft tissues, the regional lymphatics being commonly involved; the subcutaneous tumors break down in time to form abscesses. The subcutaneous lesions respond to surgery but infection of the tongue, "wooden tongue," is often fatal. The disease has been observed to occur in both epizootic and sporadic form. It is readily reproduced by inoculation of cattle but the microorganism is only feebly pathogenic for the usual experimental animals; massive intraperitoneal inoculation of the guinea pig produces a scrotal reaction similar to the Straus reaction (p. 602). A very few probable cases of human infection have been reported.

Actinobacillus Actinoides. This microorganism was originally isolated from the lungs of calves suffering from a chronic pneumonia[46] and later from a similar affection in white rats.[47] It is not pathogenic for other experimental animals and in fact its etiological relationship to the observed disease is open to some question.

Actinobacillus Actinomycetum-Comitans. This bacillus has been observed and isolated from human cases of infection with *Actinomyces bovis*, first by Kligler[48] in Germany, and later by Comstock[49] in England and Bayne-Jones[50] in this country. It occurs as densely packed gram-negative cocco-bacilli in contrast to the gram-positive actinomycete mycelium in the interior of the sulfur granules (see Fig. 157). It has never been reported in bovine actinomycosis and the significance of its presence is open to question.

ERYSIPELOTHRIX RHUSIOPATHIAE

Microorganisms closely related to the actinomycetes have been found to be the causative agents of swine erysipelas and a type of mouse septicemia; they also infect man and the disease produced is termed erysipeloid to distinguish it from erysipelas of streptococcal etiology (p. 371). It was thought for a time that the mouse septicemia organism isolated by Koch,[51] the organism isolated from swine by Pasteur and Thuillier[52] and by Löffler,[53] and that found by Rosenbach[54] in human erysipeloid were distinct species of a genus to which the name Erysipelothrix has been given, and they were called *Erysipelothrix muriseptica*, *Erysipelothrix rhusiopathiae* and *Erysipelothrix erysipeloides* respectively. The first of these, sometimes called *Bacterium murisepticum*, is not to be confused with *Pasteurella muriseptica* (p. 502) which also has the same synonym. Other names that have been used are *Erysipelothrix rhusiopathiae suis*, *Erysipelothrix porci*, *Bacillus erysipelatis suis*, *Actinomyces rhusiopathiae*, and the swine *rotlauf* bacillus. It is now generally agreed that these organisms are identical, or at least closely related varieties of the same species, for while

---

[46] Theobald Smith: Jour. Exp. Med., 1918, 28:333.
[47] Jones: Jour. Exp. Med., 1922, 35:361.
[48] Kligler: Centralbl. f. Bakt., I Abt. Orig., 1912, 62:191.
[49] Comstock: Brit. Jour. Exp. Path., 1920, 1:197.
[50] Bayne-Jones: Jour. Bact., 1925, 10:569.
[51] *Cf.* Koch: *Investigations into the Etiology of Traumatic Infective Diseases.* New Sydenham Society, London. 1880.
[52] Pasteur and Thuillier: C. R. Acad. Sci., 1883, 97:1163.
[53] Löeffler: Arb. Kaiserl. Gesundshamte, 1886, 1:46.
[54] Rosenbach: Ztschf. f. Hyg. u. Infektionskr., 1909, 58:343.

their morphology is variable they are immunologically identical, and are recognized as a single species, *Erysipelothrix rhusiopathiae*.

Morphology and Physiology. *Ery. rhusiopathiae* occurs in two rather well-defined morphological types usually designated smooth and rough though their relationship to the S and R variants of bacterial dissociation is not clear. The smooth type appears as a small, slender, sometimes slightly curved, non-motile, non-sporulating, gram-positive rod. Long chains of bacilli and filaments, sometimes beaded and showing swollen areas, are present in smears of the rough form. Both stain readily and sometimes irregularly with deeply staining granules. Colonies of the smooth form on solid media are round, convex, amorphous, water-clear, and small, perhaps 0.1 mm. in diameter, and broth cultures are uniformly turbid. The rough form produces larger colonies with a
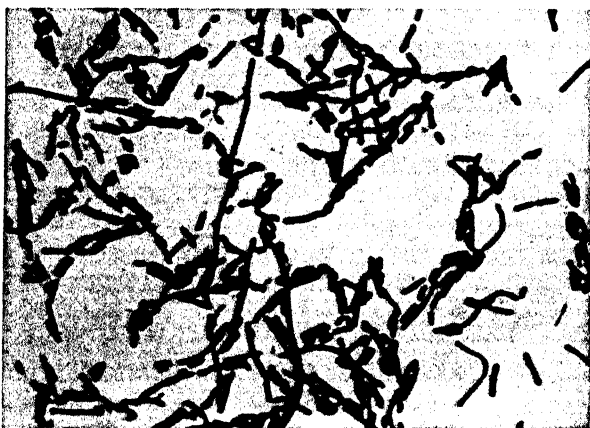
Fig. 160. *Erysipelothrix rhusiopathiae*. Pure culture. Note the similarity of this microorganism to the actinomycetes. × 1000 (Kral).

granular, curled appearance like that of very small colonies of anthrax bacilli, while growth in liquid media is in the form of flocculent, hair-like masses with little or no turbidity. The most characteristic growth is in gelatin stab cultures; bead-like colonies appear along the line of inoculation from which lateral filamentous growth occurs, resembling a test tube brush.

The organism is microaerophilic but will grow under aerobic or anaerobic conditions; growth appears on the usual infusion media with twenty-four hours' incubation at the optimum temperature of 30° C. There is no growth on potato. Fermentation reactions are variable from strain to strain but most ferment dextrose, lactose and levulose. Nitrate is reduced and hydrogen sulfide is produced, but indol is not formed and litmus milk is unchanged or slightly acidified.[55]

Ery. rhusiopathiae is somewhat more than ordinarily resistant to drying and to various preservative processes such as smoking, pickling and salting and survives for relatively long periods of time in putrefying meat and in water. It

[55] The cultural and biochemical characteristics of this organism are discussed in detail by Karlson and Merchant: Amer. Jour. Vet. Res., 1941, 2:5.

is probably in part because of survival of the organisms in filth that infected areas experience recurrences of the disease year after year.

Pathogenicity. In swine four clinical types of disease are found. In the *acute septicemic* form, with lesions in the viscera and internal organs, the case fatality rate is high, perhaps 80 per cent, with death in three to five days. The *urticarial* form, known as "diamonds" or "diamond skin disease" because of the occurrence of reddish to purplish rhomboidal blotches on the skin, may occur with or without visceral involvement and is seldom fatal. The *chronic* form is a vegetative endocarditis with erosion of the mitral valves in particular; death always results eventually. *Arthritis* may complicate the other clinical types or occur independently; it is usually not fatal but growth is stunted. The organisms are excreted in large numbers in the feces and it is generally assumed that natural infection takes place by mouth although experimental feeding has given irregular results. Infection is spread in part by healthy carriers as well as diseased animals and pork trimmings in garbage probably account for isolated cases. Swine erysipelas is of very great economic importance in Europe, especially in Germany where it is known as *Schweinerotlauf*, and in recent years has been found to be more important in this country, at least in certain areas, than formerly thought.

Sheep are occasionally infected with *Ery. rhusiopathiae* and develop a polyarthritic form of the disease. The organism is also pathogenic for a variety of birds and in this country turkeys are most often seriously affected;[56] cyanosis is a prominent clinical feature and evident in the "blue comb." It has also been found in wild rats which should, perhaps, be considered as a reservoir of infection and possibly a source of the human disease.[57]

The disease may be reproduced by the inoculation of swine though the results are irregular. Of the usual laboratory animals, white mice and pigeons are highly susceptible, dying in one to four days after subcutaneous inoculation, and are commonly used in diagnosis. The rabbit is not very susceptible and the guinea pig is quite resistant. In general the virulence of the microorganism varies widely.

Pathogenicity for Man. Human infection with *Ery. rhusiopathiae* is well known.[58] The septicemic type with diffuse erythema is rare in man and only a very few instances have been reported. The chronic form with endocarditis and polyarthritis is also very rare. The usual type of infection is an erythematous-edematous lesion, the local lesion commonly developing on the fingers or hand from an abrasion where the infection enters. The lesion, although spreading, never extends beyond the wrist. There is some swelling and a marked erythema of the region and sometimes a local arthritis and regional adenitis. The disease is usually self-limiting and terminates within a month. The organism may be cultivated from an excised piece of skin from the lesion.

Human infection can almost invariably be traced to contact with animals and animal products such as meat, hides, bones and manure, or to fish and shell-

[56] The incidence and epidemiology of the disease are discussed by Rosenwald and Dickinson: Amer. Jour. Vet. Res., 1941, 2:202.

[57] Drake and Hall: Amer. Jour. Pub. Health., 1947, 37:846.

[58] See the general discussion and review by Klauder: Ann. New York Acad. Sci., 1947, 48:531.

fish and the disease is, therefore, associated with certain occupations. For example, more than half the cases found in the Philadelphia region were in slaughterhouse workers. The disease has been observed in workers in a bone button factory using cattle and hog bones,[59] and it is not uncommon in fishermen and fish handlers. It also occurs in persons working in kitchens with raw meat and fish. In this country contact with live fish and crustacea appears to be the chief source of infection. Hettche[60] has reported finding *Ery. rhusiopathiae* in 10 of 30 specimens of sewage at Königsberg in Prussia and in 5 of 52 specimens at Munich; the source apparently was slaughterhouses where infected animals were being killed. Under experimental conditions the organisms not only survived for some days in water but fish developed a latent infection when fed infected meat; the microorganism could be isolated from most organs including the kidney, and were excreted in large numbers in the urine, contaminating the water.

Immunity. There is a definite immune response in infected hogs as evidenced by the formation of agglutinins which have diagnostic value. *Ery. rhusiopathiae* is immunologically relatively homogeneous but serological groups may be distinguished by reciprocal absorption.[61] Antiserum has therapeutic value and in man is given both intramuscularly and by infiltration about the local lesion. Passive immunization of swine is an effective prophylactic but the immunity lasts no longer than two weeks. Animals may be actively immunized with the vaccine developed by Pasteur and Thuillier[52] who attenuated the organism by passing it through rabbits. Vaccine erysipelas occurs with some frequency, however, and the method has now been largely superseded by the simultaneous inoculation of virulent culture and antiserum. Since the vaccine maintains and spreads infection in herds, the Bureau of Animal Industry has only recently (1942) permitted its limited use in this country and only in areas where the disease has become prevalent. Both methods provide adequate protection for eight to twelve months; periodic reimmunization is required for breeding stock.

II. THE MOLDS AND MOLD-LIKE FUNGI

The fungi proper, or Eumycetes, are quite distinct from the true bacteria, being closely related to the higher algae but differing from them in that they lack chlorophyll. Phylogenetically, their differentiation may have arisen through a degenerative loss of chlorophyll and adaptation to a chemosynthetic type of metabolism. Some of the fungi are unicellular, others are multicellular, and still others are sometimes unicellular and sometimes multicellular, that is to say, dimorphic; some of the parasitic fungi are unicellular in the tissues and multicellular in culture. For present purposes we shall consider the multicellular forms here under the head of molds and mold-like fungi, and the unicellular forms such as yeasts and yeast-like fungi in the following section, though the occurrence of intermediate forms and taxonomic considerations makes such a differentiation inconsistent.

[59] McGinnes and Spindle: Amer. Jour. Pub. Health, 1934, 24:32.
[60] Hettche: Arch. f. Hyg. u. Bakt., 1937, *119*:178.
[61] Gledhill: Jour. Path. Bact., 1945, *57*:179.

Hyphae and Mycelium. The multicellular fungi are made up in large part of cells attached end to end to form filaments or *hyphae*. These are considerably larger in diameter than the actinomycete filaments described earlier, and the latter are perhaps to be regarded as a rudimentary type of hyphae. In some instances certain of the hyphae may be coiled to form helices. The mass of branching, intertwining and sometimes anastomosing hyphae making up a colony of the fungus is the *mycelium*. The entire mass is also sometimes called the *thallus*.

Two main structural types of mycelium may be distinguished. In one of these the cells making up the hyphae are not separated by cross walls or septa, making possible a characteristic flowing of protoplasm through the multinucleate structure. Such a structure is said to be *non-septate* or *coenocytic*. Certain of the algae are of similar structure and the fungi thus characterized are called *phycomycetes* or algae-like fungi. In the majority of fungi, however, the hyphae are *septate*, each cell separated from the other by cross walls. The individual cells contain a single nucleus, as in the *ascomycetes*, or sometimes two as in certain of the *basidiomycetes*. Thus these three main classes of fungi may be distinguished in part on the basis of the structure of the mature, nonsporulating hyphae, though this is not one of the main characteristics upon which their differentiation is made.

The mycelium is further differentiated into two general types which differ in function. One of these, the *vegetative mycelium*, consists of masses of hyphae within the colony, adjacent to and growing into the substrate, and is concerned with the assimilation of food materials. Fragments of such mycelium will, of course, reproduce if transferred. The other type, or *reproductive mycelium*, usually extends into the air to form an *aerial mycelium* and gives rise to reproductive bodies or spores. The mode of spore formation and the structure of the spore and spore-bearing elements are the characteristics by which the fungi are differentiated, classified and identified.

It may be noted that in addition to sporulation and the vegetative growth of hyphae, many species of fungi reproduce by the separation of cells, known as *oidia*, from any part of the mycelium. These vegetative reproductive forms may give rise to new mycelium or reproduce themselves by budding like the yeasts, according to the environment in which they are placed.

Spore Formation. Two kinds of spores are to be distinguished, the *sexual spores* which are produced by the fusion of two cells which may or may not differ morphologically and which are referred to as plus and minus rather than male and female, and the *asexual spores* which arise by differentiation of the cells of the spore-bearing hyphae without fusion. Fungi for which both sexual and asexual types of spore formation are known are "perfect fungi" and are further differentiated on the basis of differences in asexual spore formation and other morphological characteristics. This group includes the phycomycetes, the ascomycetes and the basidiomycetes.

Sexual Spore Formation. The most common of the phycomycetes are species of Mucor and Rhizopus. These molds produce a sexual spore known as a *zygospore* by the fusion of neighboring filaments of the same or different plants. The ascomycetes, which include a number of genera as well as certain strains

of Penicillium and of Aspergillus, and also the yeasts, form sexual spores known as *ascospores* because they are contained in an *ascus* or sac. Their formation is simplest in the yeasts in which two contiguous cells fuse by means of minute tube-like processes, the nuclei unite, and the resulting single nucleus divides three times to give eight daughter nuclei. Reserve material accumulates about each nucleus, a spore wall is formed, and the cell containing this is the ascus. In most of the ascomycetes the process is somewhat more complex and the cells which fuse may be distinguishable and are termed the *oögonium* and *antheridium.* The cell resulting from fusion gives rise to new hyphae, the next to last cell is binucleate, and these nuclei fuse and then divide to form the ascospores, *i.e.,* two nuclear fusions are involved in the entire process. The basidiospores are also formed by a complex process including nuclear fusion; this group is made up of the mushrooms, puffballs and other fleshy fungi, none of which is pathogenic.

Fig. 161. *Rhizopus sp.* Mounted in Annam's medium. Note the single-celled (nonseptate) mycelium and ruptured, empty sporangia. The small oval bodies are free spores. The root-like structure at the base of the hyphae is the "hold-fast" by which the mold is attached to the nutrient medium. × 80.

Fungi Imperfecti. A large group of fungi, which includes practically all of the pathogenic forms, is made up of those for which a sexual phase has not been demonstrated and only asexual spores are produced. They are known as the "imperfect fungi" or Fungi Imperfecti and are sometimes called *hyphomycetes;* they make up the fourth group of eumycetes. The group is necessarily only provisional and various species of fungi are removed from it from time to time as their sexual phases are discovered. It is also heterogeneous and includes species which are much more closely related to those of other groups than to one another; for example, certain species of Aspergillus are classed with the ascomycetes while others, whose sexual phase is as yet unknown, are placed with the Fungi Imperfecti.

Differential Characteristics of Genera and Species. While sexual spore formation is the basis for primary differentiation of the eumycetes, genera and species of both the "perfect fungi" and the Fungi Imperfecti are dis-

tinguished by other morphological characteristics. These, however, assume primary practical importance, especially for the pathogenic forms. They may be summarized briefly:

(1) **Reproductive Structures.** The characteristics of asexual spore formation:

(a) In the more primitive molds, the phycomycetes, the asexual spores are characteristically produced within a capsule or *sporangium*. For example, in *Rhizopus nigricans*, the common white cottony mold found on damp bread, horse dung, etc., erect unbranched hyphae arise from the single-celled mycelium and near the apex of each a septum forms. The tip then swells into the globular sporangium, within which numerous oval spores develop; the wall of the ripe sporangium ruptures easily, and the spores are discharged by the swelling of the gelatinous mass in which they are embedded (see Fig. 161).

(b) In the higher molds the asexual spores are not enclosed in a capsule, but are produced free by segmentation of the tips of the hyphae. Such spores are known as *conidia* and the spore-bearing hyphae as *conidiophores*. Various types of structures are developed. For example:

Fig. 162. *Aspergillus sp.* mounted in Annam's medium. Note the fully developed sterigmata and the chains of conidia abstricted from the tips. The large dark masses are made up of free conidia. × 370.

(i) In Aspergillus the unbranched conidiophore arises from an enlarged cell of the vegetative mycelium, designated the foot cell, and terminates in a swollen tip, the vesicle. A number of little, tenpin-shaped stalks, the *sterigmata* or *phialides,* are given off from the vesicle and the chains of conidia are abstricted from these (see Fig. 162).

(ii) The common blue-green mold, Penicillium, has a similar structure; the terminal portion of certain hyphae breaks up into finger-like verticillate branches and, on the tips of these, sterigmata abstrict chains of conidia (see Fig. 163).

(iii) In Hormodendrum the formation of conidia differs from that of many other molds. Instead of successive conidia being formed by repeated constriction of the tip of the sterigmata to form a chain in which the terminal spore is the oldest, the conidiophore forms but one spore which buds to form a second, the second forms a third, etc. Furthermore, a spore may develop more than one bud simultaneously thus producing branching chains of conidia. It may be noted that differentiation is made among these organisms on the basis of formation of phialides.

(iv) *Aleuriospores* resemble conidia in a general way in size and shape but differ in that they are formed on short lateral branches of hyphae, and sometimes directly on the hypha itself, rather than on the tips of specialized conidiophores, and are not set free when mature but are liberated only when the mycelium that forms them disintegrates. When the lateral branches are very short with many spores and/or the aleuriospores are

directly attached to the hyphae in considerable numbers, the structures are sometimes referred to as *hyphae sporiferae, thyrsi sporiferae,* or simply as *thyrsi* (see Fig. 172). Aleuriospores are perhaps to be regarded as lateral chlamydospores (see below) and are characteristic of the ringworm fungi in particular.

(v) *Spindle-spores* or *fuseaux* are large, thick-walled structures, septate and composed of several cells, which are formed by the aerial mycelium. Fuseaux are formed in large numbers by some species of fungi and only rarely by others and assume some differential significance, especially among the dermatophytes. Some workers regard shape, blunt or pointed ends, and other morphological characters of these bodies as valid differential characteristics (see Figs. 169 and 170).

(vi) Fragmentation of the mycelium results in the production of bodies termed *arthrospores.* These are often produced by the dermatophytes in the lesions in the skin and hair but are only rarely observed in culture. They are not true spores and are perhaps best regarded as oidia in structure and function though this point is seldom made.

(c) Large, thick-walled structures formed within the vegetative mycelium, sometimes within hyphae, at others laterally on short stalks, are designated *chlamydospores* (see

Fig. 163. *Penicillium sp.* mounted in Annam's medium. Note the characteristic finger-like verticillate branches and the terminal chains of conidia. × 440.

Fig. 168). These structures are often irregular in form and some are composed of several cells and approach a spindle form. In such cases they resemble fuseaux and, in fact, in much of the literature are referred to as fuseaux.

(2) **Vegetative or Mycelial Structures.** A number of structures are formed by the vegetative mycelium which have no reproductive significance but are of considerable value in the differentiation of the pathogenic fungi.

(a) *Spirals* or coiled hyphae, similar to the coiled filaments of actinomycetes described in the preceding section, are observed in a number of the pathogenic mold-like fungi (see Figs. 171 and 173).

(b) *Nodular organs* are formed by some species. These are enlargements in the mycelium which consist of closely twisted hyphae, either side branches twining about the parent stem or different hyphae twisted together, and the resulting structure has a nobby, nodular appearance.

(c) *Raquet mycelium* or *mycelium en raquette* is a term applied to certain hyphae, usually larger than the others, that show a regular enlargement of one end of each segment, large and small ends being in apposition (see Fig. 174).

(d) The term *pectinate bodies* is applied to unilateral short irregular projections from the hyphae, giving a "broken comb" appearance.

(e) *Favic chandelier* is the name applied to the structure formed by the occurrence of numerous short multiple hyphal branches having the appearance of a reindeer horn. This

structure is not common, occurring only in the favus fungus, *Achorion schoenleinii* (see Fig. 175).

(3) **Colonial Morphology.** The morphology of colonies on Sabouraud's "differential" medium has assumed greater importance for the dermatophytes than for the other pathogenic fungi. In fact, in this group primary differentiation is made on this basis and colonial morphology often serves to identify a species.

Microscopic Examination. The methods used in the microscopic examination of the fungi vary somewhat according to the nature of the material and the purpose of the examination. In general the staining methods so useful in the study of the bacteria are not applicable; the fungi are all gram-positive and may be found in gram-stained smears but their morphology is obscured. Wet, unstained or lightly stained preparations are most informative.

Specimens. Open or draining lesions are almost always so heavily contaminated by secondary bacterial invasion that fungi are very difficult to find; in material from surgically opened lesions they are usually demonstrable though not so numerous as bacteria in corresponding bacterial infections. In the dermatomycoses secondary bacterial infection ordinarily does not interfere with the microscopic demonstration of the fungous elements. The dermatophytes live in keratin material exclusively, and specimens should be taken from scrapings of horny layers, tops of vesicles, scrapings from nail plate, and hair.

The material should be mounted in strong (10 to 20 per cent) hot sodium hydroxide, which dissolves or makes translucent the tissue elements but ordinarily does not affect the fungi, and examined as a wet unstained preparation. Antiformin or lactophenol may be used instead of sodium hydroxide. Care must be taken to distinguish between spores and fat globules and between mycelium and fibrin strands; a mycelial-like structure ("mosaic fungus") may be formed in some preparations, presumably from cholesterol. Fungi may be demonstrated in tissue sections, as the walls of abscesses, granulomatous tissue, etc., by the Unna-Pappenheim (methyl green-pyronin) stain.

Cultures. A bit of growth is removed from the colony, teased apart in a drop of water, and examined as a wet preparation. While the various structures may be seen, the arrangement of the elements is seriously disturbed. Slide cultures, such as those described by Henrici,[1] show the structure and arrangement of the growth and may be made into permanent mounts.

Cultivation. Most of the fungi grow very rapidly but the pathogenic forms usually grow relatively slowly and two or three weeks' incubation may be necessary. Their morphology is markedly affected by the type of medium on which they are grown. In general they show no unusual nutritive requirements and grow readily on all the usual bacteriological media, especially if a sugar is added. Many molds tolerate a high acidity and may be cultivated on tartaric acid-dextrose nutrient agar on which bacterial growth is inhibited. Most grow well on modified Czapek-Dox medium (a glucose-nitrate synthetic medium) which is reproducible and has been widely used as a "standard" agar for descriptive purposes. Sabouraud's medium, a peptone-maltose agar, is perhaps the most widely used medium in medical mycology for the isolation and maintenance of cultures, especially the dermatophytes. Because of the influence of the composition of the medium on morphology, it is insisted that only "crude maltose of Chanut" (4 per cent) and "granulated peptone of

Chassaing," brands obtainable only from a certain firm in Paris, be used. This is Sabouraud's "proof agar" (milieu d'épreuve). For most work, however, any maltose or peptone suffices and crude dextrose, such as corn syrup, may be substituted for the maltose.

PATHOGENIC MOLDS AND MOLD-LIKE FUNGI

Organism	Disease	Geographic Distribution
Aspergillus fumigatus	Aspergillosis—pulmonary in birds, otomycosis and pulmonary infection in man	Europe and U. S.; otomycosis most prevalent in India, probably ubiquitous
Fonsecaea pedrosoi	Chromoblastomycosis	Ubiquitous with the possible exception of Europe; especially South America and Puerto Rico
Sporotrichum schenckii	Sporotrichosis	Europe and U. S., probably ubiquitous in temperate climates
Microsporum species *Trichophyton* species *Achorion* species *Epidermophyton* species *Endodermophyton* species	Dermatophytosis in man, some species natural parasites of animals	In general ubiquitous, variable from species to species

ASPERGILLUS (ASPERGILLOSIS)

The blue-green mold commonly observed on damp bread and similar materials is usually an aspergillus, often called *Aspergillus glaucus*, though this name has been so indiscriminately applied that it now has little or no precise significance. So far as is known, these bread molds are not pathogenic. A similar mold, however, also producing a blue-green pigment and known as *Aspergillus fumigatus*, is highly pathogenic for birds and also occasionally for man.

Pathogenicity for Animals. Aspergillosis of domesticated birds, pigeons, ducks and chickens, is not uncommon and at times assumes economic importance. Three types of infection occur, infection of the air sacs, and both nodular and pneumonic forms of lung infection. Infection of the air sacs takes the form of a superficial infection of the epithelial lining, which becomes thickened and covered with a mat of green, sporulating mycelium. In the nodular form of the disease tubercle-like masses of infiltrated tissue, necrotic in the center, are formed. A diffuse infiltration develops in the pneumonic form and the lung tissue is consolidated and grayish white in color. The pneumonic disease sometimes assumes epidemic form in chicks and is known as "brooder pneumonia." The source of infection is usually moldy grain or straw. The fungus may also invade the egg during incubation with infection of the embryo. Cattle, sheep, and especially horses develop aspergillosis though less commonly than birds; the lesions are pulmonary and may be either nodular or pneumonic.

Strains vary widely in their pathogenicity when tested by inoculation of experimental animals. In general those isolated from infections are highly virulent, while those found in air and elsewhere are of low virulence. With virulent strains a fulminating, rapidly fatal pneumonia may be produced in pigeons by inhalation of spores, while feeding grain overgrown with the mold often produces an infection of the air sacs. Intravenous inoculation of pigeons results in an acute infection with multiple miliary abscesses, especially in the lungs, if the dose is not too large. Rabbits inoculated intravenously with spore suspensions of virulent strains usually die in three to five days with multiple abscess formation, notably in the kidney, while subcutaneous or intraperitoneal inoculation produces localized lesions which may or may not be fatal. Henrici[62] has shown that *A. fumigatus* produces an endotoxin.
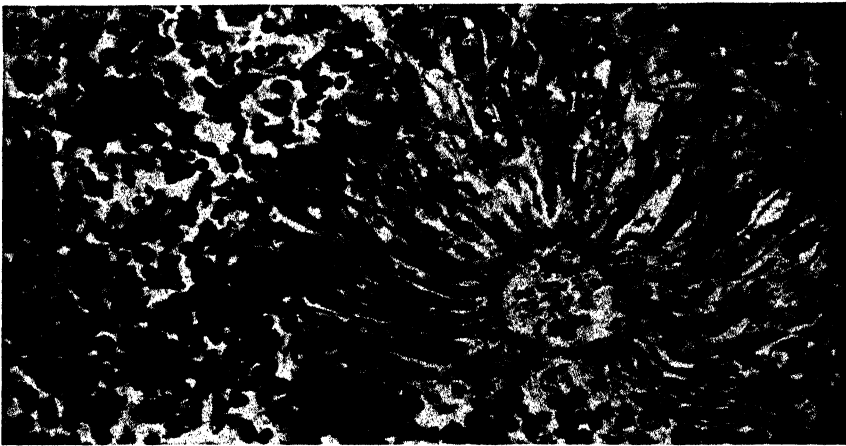
Fig. 164. Pulmonary aspergillosis in man showing a fruiting head and spores in a cavity in the lung tissue. The spores are slightly smaller than red cells and show a crenated edge when viewed from the end and a banded appearance from the side. Iron hematoxylin; × 650 (Humphreys).

Pathogenicity for Man. Human cases of aspergillosis are most frequently infections of the external ear (otomycosis). The disease varies from a plugging of the external meatus with a mass of mycelium to ulceration of the walls and even penetration of the middle ear; the mild cases are the most numerous. This type of aspergillosis is said to be relatively common in India, less so elsewhere though a considerable proportion (1 per cent) of ear infections in Germany have been reported to be aspergillosis. While *A. fumigatus* is the most common invader, *A. nidulans*, *A. flavus* and *A. niger* are sometimes found.

Infection of the lung is rare in man and probably is often secondary, particularly to tuberculosis. It is of some interest that the primary infection has been classed as an occupational disease with respect to compensation.[63] The disease is clinically similar to and may be mistaken for tuberculosis; there is extensive cavitation and it advances rapidly. The mycelium may be found in the

62 Henrici: Jour. Immunol., 1939, 36:319.
63 Coe: Ann. Int. Med., 1945, 23:324.

sputum and the fungus is readily isolated by culture but other etiologic agents must be excluded. Most of the reported cases of lung infection are among bird-fanciers, especially those having to do with the care of pigeons; there is convincing evidence that transmission of the disease from pigeons to man can take place.

Very rarely aspergillosis may take other forms in man. The occurrence of Aspergillus in maduromycosis has been noted earlier and a case of chronic suppuration with discharge of grains has been reported. It has been contended by some workers that fungi of the *A. nidulans* group may be responsible for certain types of splenomegaly but this appears doubtful.
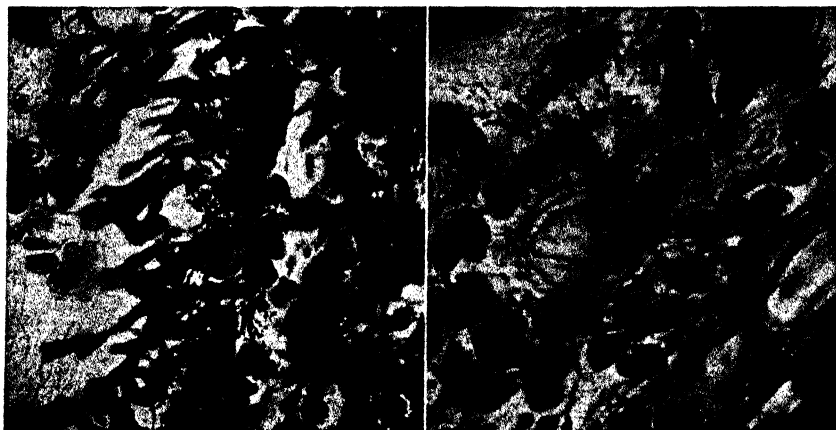
Fig. 165. Primary mycotic bronchitis in man resulting from infection with *Aspergillus sp.* Left, mycelium invading the wall of the bronchus; × 950. Right, a segment of swollen mycelium in the deeper tissues under higher magnification; × 1550. Hematoxylin and eosin (Humphreys).

FONSECAEA PEDROSOI (CHROMOBLASTOMYCOSIS)

Chromoblastomycosis was discovered by Pedroso in 1911 but his observations were not reported until 1920, the first case in the literature being reported from Boston by Medlar in 1915. The number of recorded cases is relatively small; something over one hundred authentic cases have been reported altogether. The geographical distribution is wide, however, with the greatest number of cases found in Puerto Rico and Brazil while others have been observed in other parts of South America, Africa and the Far East. Chromoblastomycosis has been of increased interest in recent years and present knowledge of the disease has been summarized by Carrion.[64]

The causative fungus is closely related to the Hormodendrum-Cladosporium group and was named *Hormodendrum pedrosoi* by Brumpt. Three types of sporulation are observed, the predominant type differing from one strain to another, and this has led to the splitting of strains into several genera with considerable confusion resulting. The new genus Fonsecaea was created by Negroni in 1936 and has gained some general acceptance and the organism is, therefore, also commonly known as *Fonsecaea pedrosoi*. Since the Hormoden-

[64] Carrion: Mycologia, 1942, 34:424.

drum type of sporulation is not an outstanding characteristic, Carrion[64] has proposed that the name *Fonsecaea pedrosoi* be accepted as the only species and that four varieties, *typicus, cladosporioides, phialophorica* and *communis*, be recognized on the basis of predominant type of sporulation. Mycelium is not formed in the tissues and the parasitic phase of the fungus is a brown sclerotic cell which divides by septation.

The disease is an infectious granuloma of the skin and subcutaneous tissues, occurring usually, though not always, on the feet and legs. It ordinarily begins with a small warty growth on the foot and extends upward through the development of satellite lesions. It almost always remains localized, metastases are very rare, and there are no constitutional symptoms. The disease develops very slowly; usually the case is of ten to fifteen years' duration at the time of examination, and some are known to have persisted for as long as forty years. In advanced cases there is some elephantiasis of the affected limb and great numbers of lesions. These vary somewhat and are of four general types: hard, elevated, pigmented nodules; large, cauliflower-like, prominent tumors; moderately elevated, dull red, scaly patches; and discrete or diffuse verrucous hyperkeratotic growths. The lesions are readily traumatized and the disease may be complicated by secondary bacterial infection and ulceration. The sclerotic cells appear as spherical bodies, perhaps 12 μ in diameter, with a thick membrane and granular protoplasm and often show internal septation. They may be observed in biopsy specimens either within giant cells or free in the tissues, and are demonstrable in the epithelial debris obtained by scraping the lesions. The fungus may be cultured from such scrapings or from infected tissue.

There appears to be no evidence of spread of the infection from one person to another. Since the disease tends to occur on the legs and feet of barefoot, outdoor laborers in the tropics, it seems probable that the fungus is a saprophyte normally present in soil or decomposing organic matter which, when introduced into the skin by a splinter or thorn or through some minor abrasion, may at times assume pathogenicity.

SPOROTRICHUM SCHENCKII (SPOROTRICHOSIS)

Sporotrichosis was first recognized by Schenck in this country in 1898[65] and a few years later in France by Beurmann and Ramond.[66] It has since been found all over the world, though the majority of reported cases are from the United States, especially from the Mississippi and Missouri Valleys, and from France. The disease, together with blastomycosis, is perhaps the most common and most important of the serious mycoses.[67]

Fungi of the genus Sporotrichum are characterized by the production of pear-shaped conidia directly from the mycelium which arise both laterally and at the tips of hyphae. The characteristic arrangement is not apparent in smears from cultures and the spores are usually found free, but is readily demonstrable in slide cultures. The hyphae are considerably more slender, 2 μ in diameter, than those of most molds. The character of the growth on agar is different from

[65] Schenck: Bull. Johns Hopkins Hosp., 1898, 9:286.

[66] Beurmann and Ramond: Ann. Derm. Syph., 1903, 4:678.

[67] For general discussion see Beurmann and Gougerot: *Les Sporotrichoses*. Felix Alcan, Paris. 1912; Foerster: Amer. Jour. Med. Sci., 1924, 167:54; *ibid.*, Jour. Amer. Med. Assn., 1927, 87:1605.

that of other molds; the colony is first soft and creamy in consistency, becoming more firm as the culture grows older, and there is no cottony mass of aerial mycelium. The growth also becomes darker with age, being at first a light tan which deepens to a dark brown or even almost black. Mycelium is not formed in the tissues and the parasitic phase of the fungus is a cigar-shaped body resembling an elongated yeast cell, 1 to 3 μ in breadth and 2 to 10 μ in length. These bodies are usually found within the leucocytes and apparently reproduce by fission. They are not spores.

The organism described by Schenck was named *Sporotrichum schenckii* by Hektoen and Perkins.[68] That isolated in France was supposed to be a different species and came to be known as *Sporotrichum beurmanni*. The differences between the two are pigmentation, *S. schenckii* being the lighter, the formation of fewer lateral spores by *S. schenckii*, and the fermentation of sucrose but not
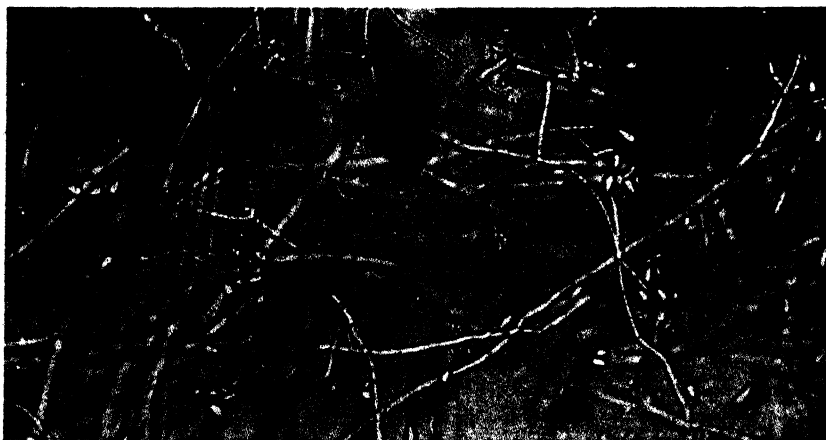
Fig. 166. *Sporotrichum schenckii* in culture. Spores and hyphae. Unstained preparation; × 450 (Rothman).

lactose by *S. beurmanni* and the reverse by *S. schenckii*. There also appear to be some differences in the clinical type of disease produced. These differences are not constant, however, and are subject to environmental modification, and it is apparent that the two are but a single species, *S. schenckii*. The name *S. beurmanni* continues to persist in the literature, however, giving the impression of two recognized species. The fungus infecting horses, *Sporotrichum equi*, is also identical with *S. schenckii*.

Pathogenicity for Man. The most common form of sporotrichosis in this country is cutaneous; the primary lesion, appearing at the site of some minor injury, often on a finger, fails to heal, ulcerates and is followed by the appearance of a series of subcutaneous abscesses along the course of the regional lymphatics. The subcutaneous lymph vessels can often be traced as reddened lines. Infection seldom extends beyond the regional lymph nodes and cases of generalized hematogenous infection are rare in this country though apparently more common in France. Metastatic lesions may appear in the liver or lungs

[68] Hektoen and Perkins: Jour. Exp. Med., 1900, 5:77.

and are most common in the testicles. The firm nodules in the skin are sug-
gestive of syphilitic gummata and probably some cases of sporotrichosis have
been so diagnosed. Ocular sporotrichosis also occurs but is quite uncommon.[69]
Henrici[1] has pointed out the common use of iodides in the treatment of tertiary
syphilis in this connection and suggests that some cases of mistaken diagnosis
may be apparently confirmed because of the therapeutic efficacy of these
compounds in sporotrichosis.

Epidemiology. Direct transmission of the infection from man to man
has been observed but is very rare. A certain number of human infections
have also been contracted, either directly through bites or indirectly by con-
tact, from naturally infected horses and rats. In the great majority of cases,
however, the fungus is introduced into the tissues from plants through some
abrasion. Thus in fourteen of eighteen cases reported by Foerster[67] infection
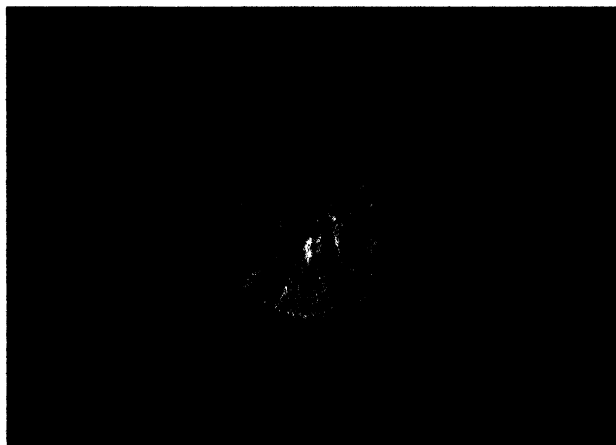
Fig. 167. Giant colony of *Sporotrichum schenckii* on Sabouraud's agar, three weeks old.
× 2.

followed wounds by barberry thorns. The disease has been observed in florists
and the fungus isolated from sphagnum moss.[70] It has been observed growing
free on grains and Benham and Kesten[71] demonstrated its ability to grow on
barberry and carnations; in the latter a bud rot was produced, an interesting
example of infection of hosts as diverse as plants and man. It seems highly
probable, therefore, that the fungus lives a saprophytic existence in nature,
occasionally setting up an infection in man when mechanically introduced
into the tissues.

Diagnosis. The diagnosis of sporotrichosis is established by demonstration
of the fungus. The cigar-shaped parasitic cell is gram-positive and may be
found in gram-stained pus smears. These forms are relatively infrequent in
human material, however, and sporotrichosis is not excluded by failure to find
them. The fungus is readily cultivated on Sabouraud's medium from pus

[69] See Gordon: Arch. Ophthalmol., 1947, 37:56.
[70] Gastineau, Spolyar and Haynes: Jour. Amer. Med. Assn., 1941, 117:1074.
[71] Benham and Kesten: Jour. Inf. Dis., 1932, 50:437.

aspirated from unopened lesions. Rats are highly susceptible to infection and rat inoculation is of considerable diagnostic value. Male white rats are inoculated intraperitoneally; a pronounced orchitis occurs and there is a generalized peritonitis with minute nodules on all the peritoneal surfaces. The cigar-shaped cells are present in abundance in the rat lesions and may be found readily in gram-stained smears.

Immunity. There is an immune response to infection manifested by the appearance of agglutinins (for spores) and complement-fixing antibodies. These are of some diagnostic value though somewhat non-specific in that sera from persons with thrush or actinomycosis will give positive reactions. A cutaneous test with *sporotrichin,* a preparation analogous to tuberculin, is of similar specificity.

THE DERMATOPHYTES[72]

By far the most common type of fungous disease of man is dermatophytosis (dermatomycosis), a superficial infection of the keratinized epidermis and keratinized epidermal appendages, the hair, hairsheaths and nails, the severity of which is dependent for the most part upon the location of the lesion and the species of fungus involved. Though certain other fungi, notably Monilia, produce clinically similar disease, a more or less homogenous group of fungi, the dermatophytes, is responsible for the great majority of cases. The ability of these microorganisms to invade and parasitize the cornified tissues is closely associated with, and dependent upon, their common physiological characteristic, the utilization of the highly insoluble sclero-protein keratin.[73] The utilization of keratin is biologically rare and is shared by the dermatophytes only with a few species of saprophytic fungi and certain insects including the clothes moth (Tinea), the carpet beetles (Dermestes) and the biting lice (Mallophaga).

Though the various species of dermatophytes produce infections that are clinically characteristic, on the one hand single species may produce different types of disease, and on the other, infections that are very similar or essentially identical may be produced by different species. Furthermore, other conditions such as chemical dermatitis, neuro-dermatitis and certain types of allergy may closely simulate dermatophytosis, but do not, of course, respond to treatment with fungicides. Demonstration of the causative fungus by direct microscopic examination of pathological material or by isolation and culture is, therefore, highly desirable, especially in all cases presented for treatment.

The dermatophytes are mold-like fungi grouped with the Fungi Imperfecti; ascospore formation has been reported by some workers but has not been unequivocally established. These organisms differ from most other pathogenic fungi in that the cells are multinucleate, usually containing 4 to 6 nuclei, and division is amitotic. Arthrospores, chlamydospores and the individual cells of fuseaux are also multi-nucleate, but aleuriospores contain but a single nucleus.[74]

[72] These organisms have been reviewed by Tate: Biol. Rev., 1929, 4:41, and by Gregory: Biol. Rev., 1935, 10:208. Later papers are discussed by Emmons: Bot. Rev., 1940, 6:474.

[73] For studies on the metabolism of these fungi cf. Goddard: Jour. Inf. Dis., 1934, 54:149.

[74] Cf. Grigoraki: Ann. Sci. Nat. Bot., 1925, Ser. 10, 7:165.

THE COMMON DERMATOPHYTES

			Species	Disease in man	Geographic Distribution
Invading the hair and hair follicles	Small spore varieties		*Microsporum audouini*	Prepuberal ringworm of the scalp; suppuration rare	Commonest in Europe, producing about 90% of infections; about half infections in U. S.
			Microsporum lanosum	Prepuberal ringworm of scalp and glabrous skin; suppuration not infrequent; kerion occasional	Uncommon in Europe; responsible for about half the infections in U. S.
			Microsporum gypseum	Prepuberal ringworm of the scalp and glabrous skin; suppuration and kerion common	Relatively rare in U. S.; common in South America
	Large spore varieties	Endothrix type	*Trichophyton acuminatum*	Ringworm of the scalp and smooth skin; onychomycosis; commonest in children but persists into adult life	Common in Europe; relatively rare in U. S.
			Trichophyton crateriforme	Ringworm of the scalp and smooth skin; onychomycosis	Common in Europe; uncommon in U. S.
			Trichophyton violaceum	Ringworm of the scalp and smooth skin; sycosis; onychomycosis; suppuration common, the hair follicles are atrophied	Common in Russia, Poland Italy, Near East, but uncommon in U. S.
			Achorion schoenleinii	Favus, in both scalp and smooth skin; onychomycosis; suppuration is the rule and kerion frequent	Common in Europe and the Far East; rare in U. S.
		Neo-endo-thrix type	*Trichophyton cerebriforme*	Usually ringworm of the glabrous skin and beard; ringworm of the scalp less frequent, onychomycosis	Common in Europe; uncommon in U. S.
		Ectothrix type	*Trichophyton mentagrophytes*	Commonest cause of intertriginous dermatophytosis of the foot ("athlete's foot"); ringworm of the smooth skin, suppurative folliculitis in scalp and beard	Ubiquitous
			Trichophyton rosaceum	Sycosis is the most common lesion; infection of smooth skin and nails	Common in Europe; sporadic distribution in U. S.
Not invading the hair and hair follicles			*Epidermophyton floccosum*	Cause of classic eczema marginatum of crural region; causes minority of cases of intertriginous dermatophytosis of foot; not known to infect hair and hair follicles	Ubiquitous, but more common in tropics
			Epidermophyton purpureum	Psoriasis-like lesions of smooth skin; onychomycosis, mild suppurative folliculitis in beard	Common in Far East, tropics and southern U. S.
			Endodermophyton concentricum	Commonest cause of tinea imbricata; infection of hair and nails uncertain	Common in South Pacific islands, Far East, India, Ceylon; reported in South America; does not occur in U. S.
			Malassezia furfur	Cause of pityriasis versicolor	Ubiquitous

The group is a homogeneous one, immunologically as well as morphologically and physiologically, and the fungi comprising it are more closely related to one another than to other fungi. The affinities of the group to the other fungi are not clear, however, and the dermatophytes have never been satisfactorily

oriented in a general system of fungus classification; they constitute a group somewhat apart and are usually neglected by botanists.

Differentiation of Genera and Species. The differentiation of species within the group is somewhat uncertain in a general sense. The classification of Sabouraud, presented in 1910[75] and somewhat modified by him later,[76] is by far the most generally used. It is based on both the clinical character of the infection and on colonial morphology, including pigmentation, the morphology of the reproductive and vegetative structures being a secondary matter. The species so differentiated are sound natural groups in spite of objections raised by some systematists and the classification and differentiation is practical and workable.

It is to be noted, however, that colonial morphology is readily altered by continued culture on artificial media. In consequence many stock cultures of

Fig. 168. Chlamydospores, *Microsporum audouini*. Mounted in Annam's lactophenol cotton blue solution; × 420.

dermatophytes are atypical to a greater or lesser extent. Such variation is especially prone to occur on maltose "proof" agar and Sabouraud recommends a *milieu de conservation*, the same medium but lacking sugar, which does not bring out the differential characters of all species; growth on it is much slower, but it has the virtue of indefinitely postponing pleomorphic changes. Differential characteristics reappear, of course, on transfer to the maltose *milieu d'épreuve*.

Sabouraud described 45 species of dermatophytes in 1910 and new species have been described more or less continuously since. According to Gregory[72] at least 184 species have been reported as pathogenic for man by 1935, and Emmons[72] states that there are at least 200 species named in the literature. In many instances the differences on which these have been based are minor and it is probable that an appreciable proportion of these new species are no more than varieties and have little practical significance. Here we shall consider

[75] Sabouraud: *Les Teignes*. Masson et Cie., Paris. 1910.
[76] Sabouraud: Ann. Derm. Syph., 1929, Ser. 6*, 10:236.

only the more important which account for the vast majority of the dermato-phytoses.

Differential Characteristics. It will be apparent that the primary differentiation of these organisms is made on a clinical basis, *i.e.*, division into the group
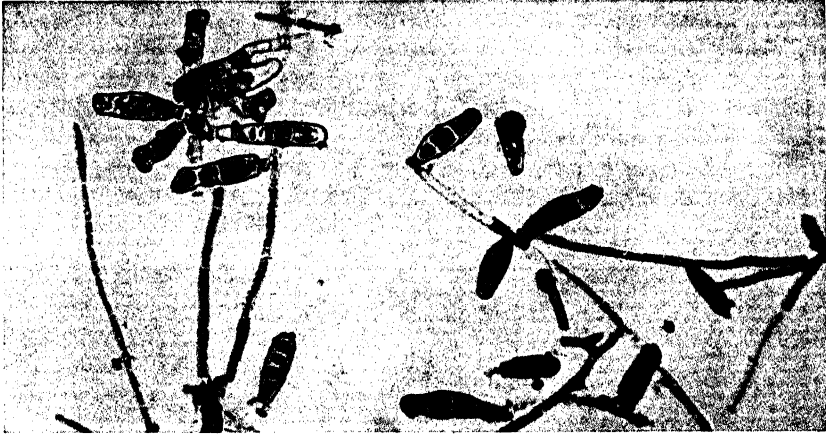
Fig. 169. Blunt end fuseaux, *Epidermophyton floccosum.* Note the attachment to the mycelium. Mounted in Annam's lactophenol cotton blue solution; × 420.

of fungi which invade the hair and hair follicles, and the group of those which do not. While parasitic on the skin and its appendages, the thallus is differentiated only into hyphae and arthrospores; the latter, arising by fragmentation of the mycelium, are to be regarded as oidia as indicated earlier though

Fig. 170. Pointed fuseaux, *Microsporum gypseum.* Mounted in Annam's lactophenol cotton blue solution; × 420.

usually termed "spores" by dermatologists. The variety of differentiated structures, including aleuriospores, spindle-spores or fuseaux, and vegetative structures such as spirals, pectinate bodies, nodular organs and raquet mycelium, appear on cultivation on artificial media.

In the first group there are, for present purposes, two genera which differ with respect to size and arrangement of the spores formed in the tissues. The genus Microsporum includes the small spore type (3–4 μ in diameter), and the genus Trichophyton, the large spore type (7–8 μ in diameter). This distinction is not absolute, however, for the very common species, *Trichophyton mentagrophytes,* forms small spores. Furthermore, the spores differ in arrangement. While the mycelium of Microsporum grows within the hairs, spores are formed only outside of the hair and occur in irregular clusters in a kind of mosaic arrangement. The spores of Trichophyton, on the other hand, occur in chains inside or outside the hair.

Further differentiation of Trichophyton is made on the basis of location of the growth with respect to the hair. The *endothrix type* (sometimes called Endotrichophyton) grows within the hair, and mycelium and chains of spores
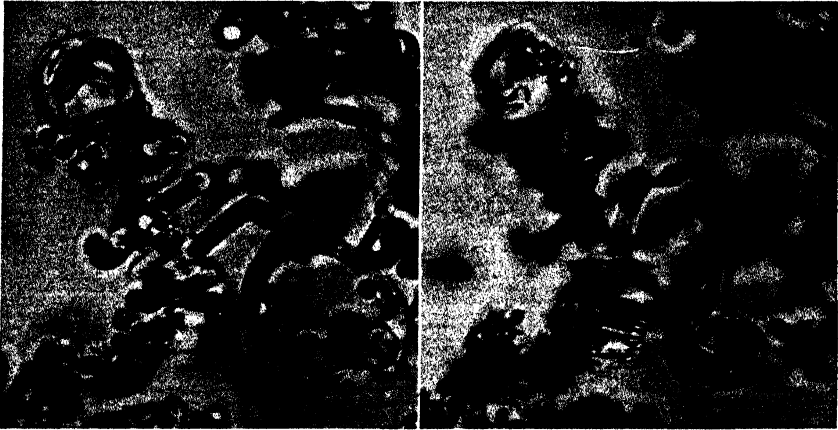
Fig. 171. Spiral hyphae, *Trichophyton mentagrophytes* var. *granulosum.* The same spiral appears in different planes of focus. The small dark bodies are microconidia. Mounted in Annam's lactophenol cotton blue solution; × 950.

are found there. The *neo-endothrix type* grows for the most part within the hair but in some hairs growth occurs on the surface. Finally, the *ectothrix type* (sometimes called Ectotrichophyton) grows only on the surface and the hyphae and chains of spores are found there. The genus Achorion is distinguished from Trichophyton by the production of yellow crusts or scutula (see below) on the scalp. It is an endothrix type and many workers believe that it should be included in the genus Trichophyton.

It will be clear that differentiation of these genera can be closely approached by, first, the clinical character of the disease and, second, by direct microscopic examination of epilated infected hairs; but identification is possible only by culture. In general Trichophyton infections show a characteristic tendency to produce an inflammatory reaction with deep infiltration of the skin that is rarely produced by Microsporum infections; this difference is of some value in distinguishing between Microsporum infections and infections with small-spore ectothrix Trichophyton. The animal strains of Microsporum such as *M. lanosum,* however, also elicit inflammatory reactions.`

Three genera of dermatophytes which do not invade the hair are considered here. Species of Epidermophyton invade the superficial layers of the skin and the fungi are found in scales of the epidermis taken from the periphery of the lesion as articulated filaments of mycelium breaking up into chains of round to oval arthrospores. Endodermophyton characteristically grows within the epithelium between the superficial and deeper layers but does not invade the corium; these forms are very difficult to cultivate. Malassezia is somewhat apart from the other dermatophytes and is not well known (see below) though the clinical disease is characteristic.

Pathogenicity. At present the dermatophytes are known only as parasites and are usually assumed to be obligate parasites either of man alone or of man and animals. The majority grow readily on laboratory media and also grow on

Fig. 172. *Hyphae sporiferae* (*thyrsi sporiferae, thyrsi*) in culture of *Trichophyton purpureum*. The attachment of aleuriospores, directly or by very short stalks, to the hyphae is evident. Unstained preparation; × 450 (Rothman).

such substrates as cereal grains, shed hair, horn debris, sterilized fragments of straw in moist tubes, etc., and will remain viable in litter containing such materials for two to three years. If protected from dryness they may live on the wooden floors of shower rooms, dressing cabins, mats, etc., for a considerable period of time. It has, therefore, been urged by some that the dermatophytes may live a saprophytic existence in nature, unrecognized because of the regular formation of a variety of differentiated structures. In any case, a saprophytic stage in the transmission from one animal to another seems possible—Davidson and Gregory[77] have suggested the shedding of infected hairs and scales—and is suggested by the findings of Muende and Webb[78] who cultured two species of Trichophyton from dung in a shed occupied by infected calves.

The dermatophytoses show, in many instances, a pronounced age and sex distribution and there is some difference in the geographical distribution of the various species. Common ringworm or "gray patch" of the scalp is confined to the young, occurring more often in boys than in girls, and is rare after

[77] Davidson and Gregory: Nature: 1933, *131*:836; Canadian Jour. Res., 1934, *10*:373.
[78] Muende and Webb: Arch. Derm. Syph., 1937, 36:987.

puberty. Others, such as *Epidermophyton* species, occur for the most part in adult males. The distribution of intertriginous dermatophytosis of the feet, commonly known as "athlete's foot," in the young adult male is probably in large part an expression of risk. *Microsporum lanosum* is more common in this country than in Europe and the reverse is true of *Achorion schoenleinii*, while *Endodermophyton concentricum* is well known in the tropics and certain parts of the Far East but is rare in temperate climates.

Some dermatophyte species appear to be so closely adapted to man that they are unable to infect lower animals while others not only produce infections in experimental animals, but animals such as the cat and dog are natural hosts and human infection may be acquired from them. There is also a high degree of specificity as to the tissues attacked. While, as indicated earlier, these fungi
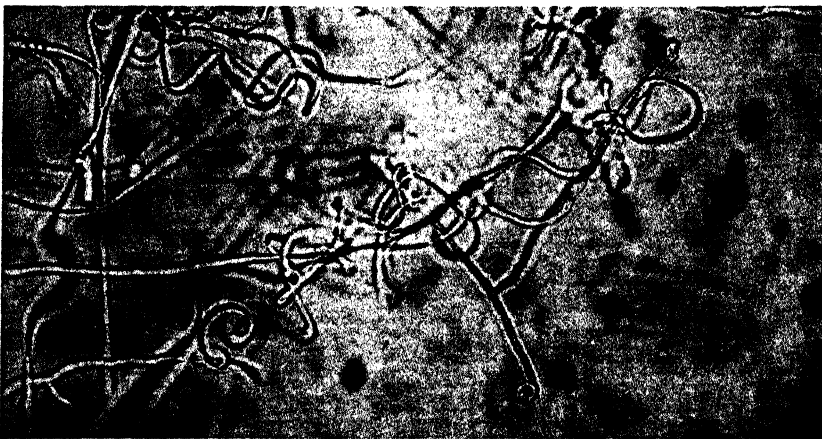
Fig. 173. Spiral hyphae in culture of *Trichophyton mentagrophytes* var. *gypseum*. Unstained preparation; × 450 (Rothman).

are well adapted to parasitize the horny layer of the epidermis, they appear to be unable to invade and infect other organs of the body. The intravenous injection of Achorion or Microsporum spores or emulsions of virulent *Trichophyton mentagrophytes* does not produce an infection of the internal organs of susceptible animals; rather, the microorganisms introduced tend to become localized in the skin and to develop where it is damaged as by scarification.

Growth of the fungus in the skin and hair is more or less equal in all directions and the lesions produced tend to have a circular form. For this reason the Greeks named the disease herpes, a term which still persists though modified as *herpes tonsurans, herpes circinatus,* or *herpes desquamans* to distinguish the dermatophytoses from herpetic infection of virus etiology, herpes simplex in its various manifestations, and herpes zoster of as yet unknown etiology (*cf.* p. 862). The Romans associated the lesions with lice and named the condition *tinea,* meaning any small insect larva. This name is likewise in common use. The English *ringworm* is, of course, a combination of the Greek and Roman terms.

With any species of dermatophyte, infection begins in the horny layer of

the epidermis. Those which infect the hair follicles, hair and nails soon invade these structures, often producing little more than a scaling of the epidermis. Those which remain in the epidermis affect the drier parts of the skin including the palmar and plantar surfaces, or the moister regions in the inguinocrural fold and the interdigital spaces. Thus a wide variety of clinical types may be observed, but the differences are more apparent than real for the pathologic changes are fundamentally the same in all types.

Infection of the Dry Glabrous Skin. The formation of characteristic concentric rings of inflammatory reaction is more evident in infections of the smooth skin than in hirsute areas. Two types of lesion are produced, the one dry and scaly, and the other vesicular. The first begins as a small elevated area of inflammation which spreads, the margin remaining red and sometimes slightly swollen while the central area becomes covered with small scales; spontaneous healing occurs in the center as the circinate margin advances.

Fig. 174. Chlamydospore (upper left) and raquet mycelium (lower right) in culture of *Microsporum audouini*. Unstained preparation, oblique lighting; × 950 (Rothman).

In the second type vesicles appear irregularly or immediately back of the advancing hyperemic and elevated margin; a crust is formed, followed by spontaneous healing in the center to leave a more or less pigmented area. The lesion may become pustular with invasion of the hair follicles. In either case the fungi are most numerous at the margin of the advancing lesion, and scales or vesicles from this region are best suited for study and isolation of the microorganism.

Infection of the palmar and plantar surfaces (the latter is the more common) is ordinarily secondary to infection of the interdigital spaces. Vesicles are formed with the thick horny layer of the sole separated from the epidermis beneath; the vesicles may be filled with fluid but soon dry. When the top peels off it leaves a pink area surrounded by a border of loosened and peeling skin. The formation of secondary vesicles and continuous repetition of the process produces widening areas of pink or reddish skin, the dysidrotic form of the disease. The formation of fissures, common in the interdigital areas, may also occur on the sole of the foot. A dry, hyperkeratotic type of lesion without vesiculation is produced in the chronic infections.

Infection of the Epidermis of the Principal Folds. In these regions there are also two main types of dermatophytosis, the one, *eczema marginatum*, chiefly confined to the crural fold, and the dysidrosiform intertriginous mycoses of the hands and feet. In the former a small round swollen area of inflammation appears which spreads, producing at first a circinate lesion which later becomes irregular owing to differences in rate of spread. The actively developing margin is an inflamed area in which there are numerous small

vesicles filled with a serous exudate. In most instances the infection begins on the thigh where it is in contact with the scrotum, and spreads rapidly, usually involving the inner thigh and sometimes the gluteal and pubic regions. Pruritus is a common symptom.

In the interdigital areas, usually of the toes (moniliasis is the more common in the drier areas between the fingers) the epidermis is macerated or it shows circinate scaling. Small eroded patches are common. Vesicles are rarely seen because they rupture early, leaving circular or polycyclic epithelial fringes. Deep within the fold the epidermis is membranous and swollen, fissures form and the macerated epithelium is shed as moist, white scales.

Infection of the Hair and Hair Follicles. Invasion of the hair follicles and hair produces two general types of lesions. The most distinct and first to be differentiated clinically is that of *favus*, produced by *Achorion schoenleinii* and distinguished by the formation of the *favic scutulum*. Beginning with a small spot or scaly inflammation, crusts appear as yellow points about the hair follicles. The hyphae and spores form a ring about the follicular orifice and a leucocytic infiltration produces an intraepidermic pustule. A yellow disk appears at the base of the pustule which enlarges with the lesion to produce the cup-

Fig. 175. Branched hyphae or favic chandelier of *Achorion schoenleinii*. Unstained preparation; × 600 (Rothman).

shaped yellow crust known as the scutulum, which in time often becomes as large or larger than a pea. On the upper or concave surface the epidermis is intact and the hair is brittle, lusterless and gray, but not necessarily broken, while the epidermis below is infiltrated and sclerosed. Exfoliation of the horny layer occurs, some of the upper layer of the scutulum being removed. The scutulum itself consists of a mass of fungous mycelium in an enlarged hair follicle; the center surrounding the hair is a tangle of broken hyphae and spores inside an area of regular hyphae in the epidermis, and at the periphery the hyphae are perpendicular to the surface. Infection of the hair begins at the root and some time, usually three to four months, is required for the fungus to grow up through the hair to produce the dull, powdery gray, dry appearance characteristic of favus. The hair remains, though seldom more than a few centimeters in length, for several years before being shed through scar formation in the follicle, but in time it loosens, falls out, and may not return, resulting in patches of permanent alopecia. Section of infected hairs shows air spaces, presumably left by dead hyphae; relatively few hyphae remain and the hair structure is clearly visible.

The second type of infection includes ringworm of the scalp or *tinea tonsurans*, which is much the more common in this country, and infection of the beard. It may be subdivided into Microsporum infection, endothrix Trichophyton infection, and suppurative infections usually with ectothrix Trichophyton.

In Microsporum ringworm the fungus invades the hair follicle to form a mass of mycelium between the hair and the epithelium. Some hyphae penetrate the hair and grow

downward, while others grow on its surface to form the characteristic layer of spores. The lesion begins as a small erythematous area. Within a few days it pales and becomes scaly and the hair assumes the characteristic grayish, discolored appearance, becomes weakened and breaks off a few millimeters above the scalp. The lesion spreads in the characteristic

ASSOCIATION OF DERMATOPHYTE SPECIES WITH CLINICAL CHARACTER OF THE INFECTION

(1) Lesions of the smooth skin:
    (a) Lesions of the moist epidermis of the principal folds:
        (i) Eczema marginatum, usually confined to the crural region:
<div align="right">EPIDERMOPHYTON</div>

        (ii) Dysidrosiform intertriginous mycoses of the feet and sometimes hands:
<div align="right">EPIDERMOPHYTON
ectothrix TRICHOPHYTON</div>

    (b) Circinate lesions on the backs of the hands, arms, face, neck and trunk:
        (i) Intricate pattern of concentric rings—tinea imbricata:
<div align="right">ENDODERMOPHYTON</div>

        (ii) Reddish patches, not raised, with actively advancing margin and a tendency to central healing:
<div align="right">usually MICROSPORUM
sometimes endothrix TRICHOPHYTON</div>

        (iii) Reddish, elevated, scaly plaques, usually pustular at the margin:
<div align="right">ectothrix TRICHOPHYTON</div>

(2) Lesions of the hair and hair follicle:
    (a) Scutula present, hairs tend to split longitudinally and not break off transversely:
<div align="right">ACHORION</div>

    (b) No scutula present, hairs tend to break off transversely:
        (i) No suppuration, as follicular abscess, pustules, kerion:
            1. Hairs broken off at uniform height several millimeters above the skin, scaling, spores in irregular clusters on the outside of hairs:
<div align="right">MICROSPORUM</div>

            2. Hairs usually broken off flush with the follicular orifice, little scaling, mycelium and chains of spores within the hair:
<div align="right">endothrix and neo-endothrix TRICHOPHYTON</div>

        (ii) Suppuration, lesions of smooth skin often present, fungus both in and on the hair, spores largely external in chains:
<div align="right">ectothrix TRICHOPHYTON</div>

(3) Lesions of the nails, no distinctive clinical features:
<div align="right">ectothrix TRICHOPHYTON
EPIDERMOPHYTON
ACHORION</div>

ring form and may coalesce with other infected areas produced by autoinoculation to give an irregular area of infection in which all the hairs are affected. There is little inflammatory reaction.

Trichophyton ringworm differs from the Microsporum infection in a number of respects. While infection of the hair follicle and hair follows essentially the same course, the lesions are small with often only two or three affected hairs and all the hairs within a given lesion are not affected, and the lesions are multiple, numerous and scattered over the scalp. The infected hairs break off sharply at the follicular orifice in *Trichophyton acuminatum* infections, and somewhat above to leave stumps in *Trichophyton crateriforme* infections. There is somewhat more inflammatory reaction than in microsporiasis, and often spread of the infection to the glabrous skin.

Infections with ectothrix Trichophyton are distinguished clinically by a more marked inflammatory reaction and often suppurative folliculitis. There is an infiltration of serum and leucocytes into the skin of the affected area and an infiltration of the deeper tissues. In areas of contiguous suppurative folliculitis an indolent cutaneous-subcutaneous infil-

tration called *kerion* is formed which is boggy on palpation, pus oozing from the follicles on pressure. Suppurative lesions of the beard are termed *sycosis*. Suppurative infection and kerion may, of course, occur with other species, including endothrix Trichophyton and Microsporum of animal origin.

It was observed by Margarot and Devéze[79] in 1925 that infected hairs and fungus cultures show fluorescence in ultraviolet light.[80] This empirical observation has proved to be of very considerable practical value, especially in Microsporum ringworm of the scalp. It is generally agreed that all hairs infected with Microsporum show a brilliant greenish fluorescence and those infected with *Achorion schoenleinii* a greenish but less brilliant fluorescence, both distinct from the bluish tint of normal skin. There appears to be some disagreement with respect to Trichophyton infection; it is reported by Davidson and Gregory[81] that in infection with some species the hairs fluoresce but in others they do not, while Lewis and Hopper[1] state that all endothrix Trichophyton species show a dull bluish fluorescence of the infected hair though hair infected with ectothrix Trichophyton does not fluoresce. Davidson and Gregory[81] have found that the fluorescence is due to the presence of a substance in the hair which can be extracted either with warm water after a preliminary ether extraction, or with dilute alkali.

Infection of the Nails. Infection of the nails, *tinea unguium* or *onychomycosis*, may be either primary or secondary to an epidermal lesion. The infected nails are usually opaque, lusterless, yellowish and friable and varying degrees of dystrophy are found. The infection ordinarily begins under the free border or along the lateral margin of the nail, producing an opaque, yellowish-white irregular spot beneath the central area. This may develop into a thick spongy friable layer, readily detachable. As the nail plate separates from the bed, yellowish or white longitudinal streaks usually appear, it becomes thin and brittle and breaks off; sometimes the entire nail plate is lost. When the surface of the nail is attacked early, it becomes fissured and soft and wears away. In general, there is no marked inflammatory reaction. The disease is of long duration, and may last ten years or more, and there is little if any tendency to spontaneous healing. The disease is usually due to Trichophyton but Microsporum and Epidermophyton infections have been observed.

Immunity. Though an acquired immunity to infection has been demonstrated in experimental animals by a number of workers, the status of an effective immunity in man is uncertain. There have been some enthusiastic reports of the efficacy of vaccine therapy which are, perhaps, open to question. Much of the work that centers about local immunity appears to be uncritical, *viz.*, in many cases remarkable results have been reported but have not been regarded by immunologists generally as contributing materially to a solution of the general problem of local immunity (p. 324). Hypersensitivity, however, is a common, though not invariable, manifestation of the immune response to dermatophyte infection, and desensitization procedures appear to have definite therapeutic value in certain cases.

Hypersensitivity is manifested in two ways. One of these is the appearance of secondary, non-parasitic lesions on parts of the body remote from the infection. These are termed "ids," in a general sense *mycid* or *dermatophytid*, and more specifically *microsporid, trichophytid* and *epidermophytid*. The mycid takes the form of a symmetrical eruption over relatively large areas, usually of the trunk, as a rash. The eruption may be vesicular with sterile content, papular, or lichenoid, and is sometimes localized at the follicular pores. A rather frequent occurrence is a sterile vesicular eruption on the hands secondary to

[79] *Cf.* Margarot and Devéze: Ann. Derm. Syph., 1929, Ser. 6°, *10*:581.

[80] "Black light," commonly known as Wood's light because the radiation is filtered through Wood's nickel oxide glass which holds back almost all the visible rays but passes the longer ultraviolet rays.

[81] Davidson and Gregory: Canadian Jour. Res., 1932, 7:378.

infection of the feet. It is generally believed that spores or bits of mycelium from the infection lesion enter the blood stream and are eventually deposited in the skin where they induce the local allergic response with destruction of the fungous elements. This view is based in part on a number of successful isolations of these fungi from the blood stream during the development of mycids. It is not known what part, if any, soluble substances liberated by the dissolution of the fungi play in the phenomenon.

Hypersensitivity may also be demonstrated by the injection or application of preparations of dermatophyte cultures analogous to tuberculin and called *trichophytin*. The local and constitutional reactions that may be produced with trichophytin are much the same as those to tuberculin. The test does not differentiate between Microsporum and Trichophyton or their species for all appear to contain a common or very closely related antigen. The utility and reliability of the trichophytin test in diagnosis is a subject of conflicting opinion. It is maintained by some, such as Wise and Wolf[82] and Lewis, MacKee and Hopper,[83] that the test is a valuable adjunct to other methods of diagnosis when used with the proper precautions; others, however, such as Swartz,[1] regard the test as of questionable value because, on the one hand, infection does not always result in sensitization, and, on the other, a positive reaction may only indicate a past infection unrelated to the condition in question.

Characteristics of Dermatophyte Species. The characteristics of the more important species of dermatophytes may be considered briefly:

Microsporum audouini (*Sabouraudites audouini, Closteroaleuriosporia audouini*): This fungus is the classic etiologic agent of common prepuberal ringworm of the scalp; it is relatively more common in Europe, where it causes perhaps 90 per cent of the infections, than in this country where it is found in about half the cases. An inflammatory reaction is produced only occasionally, suppuration is uncommon, and kerion very rare. The trichophytin reaction is usually slight or absent.

Microscopic examination of the epilated hair stub shows hyphae within the hair and external irregular clusters of spores. The colony on maltose agar begins as a white fluffy mass which developes a central elevation and radial furrows and becomes grayish to buff in color as it matures. Aerial growth is scanty. About two weeks' incubation suffices for full development of colonial character. In slide culture fuseaux are rare and conidia few, but arthrospores, pectinate bodies and raquet mycelium are abundant. Experimental animals are highly resistant to infection.

Microsporum lanosum (*Microsporum canis, Microsporum caninum, Sabouraudites lanosus, Closterosporia lanosa*): Microsporum felineum is regarded by some as a separate species and by others as only a variety of *M. lanosum*. The fungus is the cause of half or more of the cases of ringworm in children in this country, but is found in only a small minority of cases in Europe. It differs from *M. audouini* in that it is a natural parasite of lower animals—it was originally found in the dog—and children may contract the disease from infected kittens and puppies as well as from one another. Infections of the smooth skin are common and produce a circinate lesion with a vesicular border and a mild erythema. In the scalp there is a mild inflammatory reaction and the infection is not infrequently suppurative; kerion occurs in 2 to 3 per cent of the cases. The trichophytin reaction is usually strongly positive.

In the epilated hair the fungus is indistinguishable from *M. audouini*. Colonial growth is somewhat more rapid and a yellowish pigment is produced, the mature colony being tan and the medium a yellowish color. Both radial and concentric striations or furrows are often present and there is an abundant and wooly aerial growth. Pleomorphism after four

[82] Wise and Wolf: Arch. Dermat. Syph., 1936, 34:1.

[83] Lewis, MacKee and Hopper: Arch. Dermat. Syph., 1938, 38:713.

to five weeks' incubation is the rule. In slide culture fuseaux are abundant in the center of the growth; other structures such as conidia, raquet mycelium and pectinate bodies are not striking. Kittens, puppies, rabbits and guinea pigs are readily infected experimentally.

Microsporum gypseum (*Microsporum fulvum, Closterosporia fulva, Achorion gypseum*): This organism is present in South America and is apparently imported into this country where it is found only occasionally. Like *M. lanosum*, it is an animal parasite and is one cause of dog favus. The disease in the scalp is similar to that produced by *M. lanosum* but with more marked inflammatory reactions as a rule, and suppuration and kerion are common. The smooth skin may be infected. The trichophytin reaction is strongly positive.

In infected hairs the spores tend to a linear arrangement in the early stages of the disease, and later the picture is indistinguishable from that produced by *M. audouini* and *M. lanosum*. Colonial morphology is highly characteristic; a central umbo, which may be white, appears early and the colony is a cinnamon to pinkish brown. Concentric furrows may be present. In slide culture fuseaux are numerous, and raquet mycelium and nodular
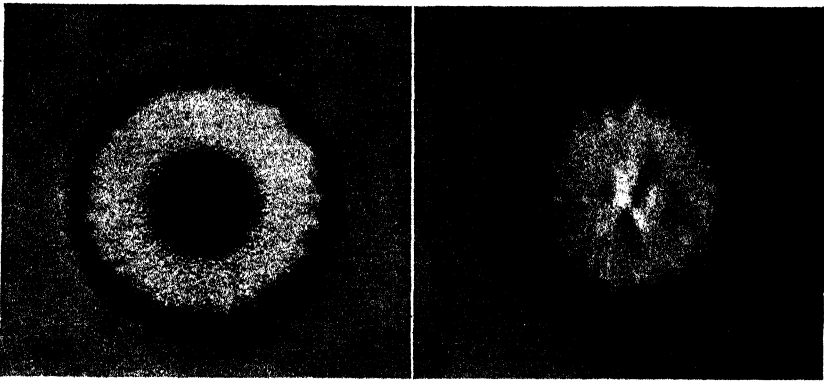
Fig. 176. Dermatophyte colonies on Sabouraud's agar. Left, *Microsporum audouini*. The colony is greyish with a buff center. Right, *Microsporum lanosum*. The colony is a light pinkish tan with a yellow border. Natural size.

organs may be found together with moderate numbers of conidia. The rabbit, cat and guinea pig may be infected.

Trichophyton acuminatum (*Trichophyton sabouraudi, Aleuriospora acuminata*): This fungus is one of the common causes of trichophyton ringworm of the scalp in Europe. Like other trichophytoses, *T. acuminatum* infections, though more common in children, persist into adult life. Infection of the smooth skin occurs with the formation of a circinate lesion with vesicular border. Infection of the nail occurs. The trichophytin reaction is positive.

The fungus is of the endothrix type and chains of spores are found within infected hairs. The colony is acuminate, *i.e.*, there is a flattened conical peak projecting from its center, with radiating furrows and peripheral folds. It is creamy white in color and the surface is fine and powdery. In slide culture there is an abundance of round to oval microconidia, borne laterally or on the tips of hyphae, as thyrses and *en grappe*. Chlamydospores are present but there is no charactertistic predominance of vegetative structures. A mild localized infection may be produced in the guinea pig.

Trichophyton crateriforme (*Trichophyton tonsurans, Chlamydoaleuriospora crateriformis*): This is the other common ringworm trichophyton found in Europe (according to Sabouraud *T. acuminatum* and *T. crateriforme* cause 72 per cent of Trichophyton infections) but is not often encountered in this country. A mild inflammatory reaction occurs in the scalp, and lesions of the glabrous skin are crusted rather than scaling. The nails may be infected. The trichophytin reaction is negative or weakly positive.

The spores present in chains within infected hairs tend to be more cylindical than round and have the appearance of a ladder rather than a string of beads. Growth is relatively slow, the colony is creamy in color and velvet-like in appearance, and is crateriform, *i.e.*, sunken in the center with a raised ring at the periphery. Morphology in slide culture is very similar to that of *T. acuminatum*. A very mild, spontaneously healing lesion may be produced by guinea pig inoculation.

Trichophyton violaceum (*Favotrichophyton violaceum, Bodinia violacea, Arthrosporia violacea*): This fungus is widely distributed over the world and is especially prevalent in Russia, Poland, Italy and the Near East. In this country it is usually, though not always, found in immigrants. The lesions on the scalp are much the same as those produced by other species of Trichophyton; the formation of small pustules and follicular crusts is common. Similar lesions are produced in the beard and the infection often spreads to the nails. The trichophytin reaction is negative or faintly positive.

In the infected hair the morphology of *T. violaceum* is that of typical endothrix Trichophyton. Colonies grow slowly and remain relatively small. The colony is of the acuminate type and in recently isolated cultures is a deep violet in color, but pigmentation is
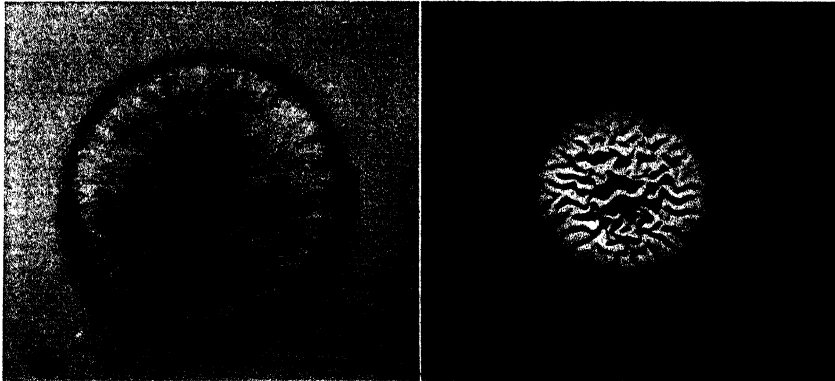
Fig. 177. Dermatophyte colonies on Sabouraud's agar. Left, *Microsporum gypseum*. The colony is a uniform pinkish tan color. Right, *Achorion schoenleinii*. The colony is a very light pinkish yellow and characteristically wrinkled.

readily lost on continued cultivation. Conidia are not formed in culture and the surface remains smooth and without a powdery coat. No characteristic structures are found in slide cultures. Guinea pigs, dogs and cats have been infected by experimental inoculation.

Achorion schoenleinii (*Trichophyton schoenleinii, Arthrosporia schoenleinii, Grubyella schoenleinii*): Favus is prevalent in Eastern Europe and the Far East but relatively uncommon in this country. It is characterized by the formation of the favic scutula described earlier. The formation of scutula is the rule on the scalp and they may be found in infection of the skin, or the skin lesions may be vesicular or scaly. Infection of the nails with *A. schoenleinii* produces severe dystrophy of the nail plates. Most human cases are infections with this species but related forms produce disease in animals, such as *Achorion quinckeanum* of mouse favus, and are occasionally transmitted to man.

Infected hairs show large spores in chains and coarse filaments within the shaft and, characteristic of this infection, air bubbles left by degeneration of the fungous elements within the hair. The distribution of the fungus in the scutulum has been described earlier. Growth in culture is slow and usually three weeks are required for development of the colonies. They are yellowish and waxy in appearance at first, and later usually become wrinkled and whitish with aerial mycelium. The fungus is highly pleomorphic in slide culture, showing various irregular bodies, swollen and distorted hyphae, etc., but the branched structure known as the favic chandelier is readily found and diagnostic, for it is not formed by other species of dermatophytes. The fungus is pathogenic for mice,

and nodular lesions of the lungs or peritoneum may be produced in the rabbit by intra-
venous or intraperitoneal inoculation respectively.

Trichophyton cerebriforme (*Trichophyton flavum, Neotrichophyton flavum*): This
fungus infects the beard and smooth skin and, more rarely, the scalp. It is common in
England, France, Germany and Italy, less so elsewhere. The lesions are indistinguishable
from those produced by *T. crateriforme*.

The location of the fungous elements in the infected hair is, for diagnostic purposes, of
the endothrix type and very similar to that of *T. crateriforme*. The young colony is white
and crateriform but as it matures the central depression tends to fill out, the folds become
prominent and it becomes yellowish in the center. The morphology in slide culture is very
similar to that of *T. acuminatum* and *T. crateriforme*. Experimental infection may be
produced in guinea pigs and the fungus has been reported to occur naturally in cats.

Trichophyton mentagrophytes (*Trichophyton gypseum, Trichophyton asteroides,
Trichophyton pedis, Trichophyton interdigitale, Trichophyton niveum, Ectotrichophyton
mentagrophytes, Sabouraudites asteroides, Aleuriospora rosacea, Ctenomyces mentagro-*
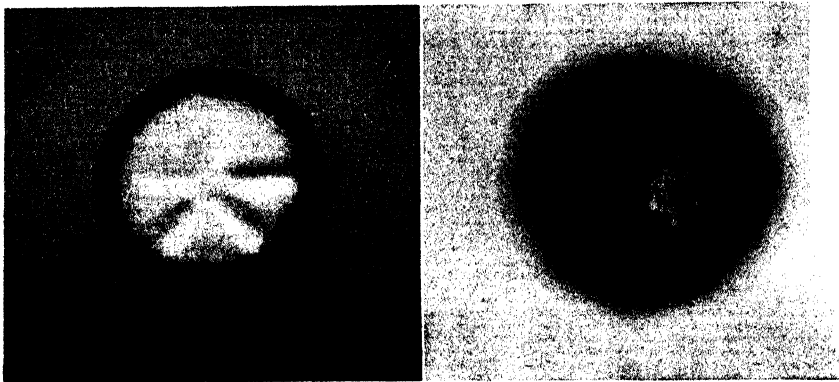
Fig. 178. Dermatophyte colonies on Sabouraud's agar. Left, *Epidermophyton pur-
pureum*. The colony itself is white with sometimes a very pinkish tinge, and the fungus
derives its name from the deep rose-purple pigment on the bottom of the colony; here the
pigment has diffused slightly to give a rim of color about the colony. Right, *Trichophyton
violaceum*. In this case the deep violet pigment is in a ring and the remainder of the colony
is rose and tan.

phytes, Spiralia mentagrophytes): This fungus is by far the commonest cause of dermato-
phytosis of the foot (athlete's foot), and also produces tinea circinata and follicular infec-
tion of the scalp with kerion as well as onychomycosis. The number of synonyms is due
in part to the pleomorphism of the fungus; the first five given above have been commonly
used in the past to denote different but closely related species. It is becoming increasingly
apparent, however, that these forms lie in continuous series between the granular *T.
gypseum* varieties and the floccose type of growth characteristic of *T. interdigitale*. Here
we shall follow the suggestion of Emmons[72] and include them all in the single species
T. mentagrophytes. It may be noted also that *Trichophyton granulosum* and *Trichophyton
felineum* are so closely related to *T. mentagrophytes* that they can hardly be regarded as
more than varieties of that species. Trichophytids associated with intertriginous infection
are usually due to this fungus. The trichophytin reaction is positive.

Infection of the hair is of the ectothrix type and, as noted earlier, the fungus is a small-
spore type. The hair is usually not broken and the external spores are relatively few in
number and in linear arrangement in contrast to Microsporum. In scales and macerated
tissue hyphae and spores are found but are not characteristic. There are four general
types of growth with respect to colonial morphology. (1) The type most often found
produces a white fluffy colony that becomes flat, velvety and yellowish-buff in color as
it matures. Slide cultures are characterized by the presence of spirals. Nodular organs,

raquet mycelium, pectinate bodies, chlamydospores and fuseaux may be found together with conidia in thyrsi and *en grappe*. (2) The white fluffy type with aerial mycelium is often called *T. interdigitale*. Fuseaux and spirals are usually absent in slide cultures; the aerial mycelium is largely vegetative with conidia in small clusters, and nodular organs and raquet mycelium may be found. (3) The granular, powdery, light tan to yellow colony is the *T. gypseum* type of growth. The surface is velvety and tends to become fluffy with age. Slide culture shows few spirals but numerous fuseaux and conidia *en grappe* and as thyrsi. (4) The white colony of the *T. niveum* type begins as fluffy but becomes compact and shows surface irregularities with maturity. The morphology of the fungus in slide culture closely resembles that of the *T. interdigitale* type. Dogs, cats, rabbits and guinea pigs may be infected; the granular types of the fungus are the more virulent.

Trichophyton rosaceum (*Trichophyton roseum, Ectotrichophyton megnini, Megatrichophyton megnini, Megatrichophyton roseum, Aleuriospora rosacea*): This fungus infects the epidermis, nails and hair follicles, usually without marked inflammation, and lesions are most common in the beard. It is common in the north of England and endemic in most
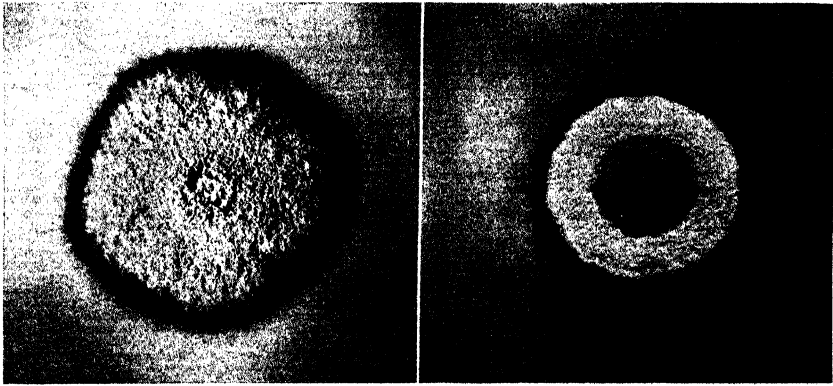
Fig. 179. Dermatophyte colonies on Sabouraud's agar. Left. *Trichophyton mentagrophytes* var. *granulosum*. The colony shown is the granular type, almost all pure white except for a slight tan cast. Right, *Epidermophyton floccosum*. The colony is a light olive drab in color with a more deeply colored area in the center.

of Europe; it has been found with some frequency in Philadelphia. The infection is spread through barber shops.

Infection of the hair is of the ectothrix type, the spores are arranged in chains and, in contrast to *T. mentagrophytes*, are large. The lesion on the skin is usually dry and scaly, and the fungous elements may be found in scrapings. The colony is velvety, pure white when young and gradually turning a pale rose with maturity while the under side becomes violet. Chlamydospores and conidia, *en grappe* and as thyrsi, are found in slide culture, but the morphology is not characteristic. Infection may be produced in the guinea pig.

Epidermophyton floccosum (*Epidermophyton inguinale, Epidermophyton cruris, Trichophyton inguinale, Trichophyton cruris, Closterosporia inguinalis, Fusoma cruris*): This fungus is the cause of classic eczema marginatum (dhobie itch) and is ubiquitous in distribution though generally regarded as considerably more prevalent in the tropics. It may be disseminated in semi-epidemic form. It is also the cause of a minority of the intertriginous dermatophytoses of the feet. This fungus has never been observed to infect the hair follicles. The trichophytin reaction is practically always negative.

In scales taken from the advancing margin of the lesion, hyphae and chains of spores may be found, in large amount when the scaling is profuse. Growth is relatively slow. The colony is characteristically an olive drab color, aerial mycelium is usually scant, and the surface is velvety and irregularly folded with radial furrows. Pleomorphic growth, manifested as the appearance of white tufts, appears relatively early. Blunt end fuseaux are present in groups in slide cultures of the fluffier strains and there are many chlamydo-

spores, but structures other than raquet mycelium are seldom found. The fungus is only slightly pathogenic for the guinea pig.

Epidermophyton purpureum (*Trichophyton purpureum*): This fungus is closely related to *Epidermophyton* (*Trichophyton*) *rubrum* and is one of the common causes of intertriginous infection of the foot. It invades the skin to produce a typical psoriasis-like lesion, mildly inflammatory with the affected area dull red and scaly and the skin thickened. It is not infrequently found in onychomycosis, and a mild suppurative folliculitis may be produced in the beard. The fungus is found in the Far East and is common in the tropics and the southern part of this country. The trichophytin reaction is negative or faintly positive.

Fungous elements are rare in infected material. The colony is at first raised, fluffy and white. Radial grooves are formed and the typical rose-purple color at the back of the colony develops early and gradually diffuses into the colony to give a white center on a tinted background. Strain variation in the formation of aerial mycelium results in con siderable variation in color as viewed from above. The morphology in slide culture is not
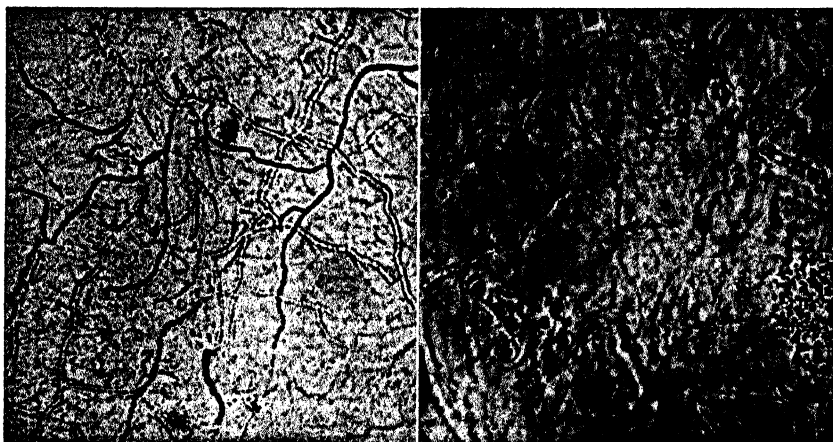
Fig. 180. Skin scrapings from lesions of dermatophytosis. Left, hyphae and spores. Right, hyphae and epithelial scales. KOH preparation; × 450 (Rothman).

characteristic, consisting largely of vegetative hyphae with occasional raquet mycelium, conidia, fuseaux and chlamydospores.

Endodermophyton concentricum (*Trichophyton concentricum*): This fungus is the commonest cause of *tinea imbricata* (tokelau, Malabar itch), a ringworm of the glabrous skin found in the South Pacific Islands, the Far East, and in India and Ceylon. Recently it has been reported from South America but apparently does not occur in this country. The closely related species, *Endodermophyton indicum* and *Endodermophyton tropicale*, are frequently found in Malaysia and Ceylon respectively. The name *E. tropicale* is used by many as a synonym of *E. concentricum*. The fungus grows between the superficial and deeper layers of the skin with little or no inflammation to produce a dry scaling lesion. The picture of the disease is highly characteristic; a circinate lesion is produced and as the margin advances, the process begins again in the center and similarly advances peripherally. With repetition concentric rings of white scales are formed. The lesions may be widespread over the body, those from different foci intermingling to produce a complex pattern. There is some disagreement as to whether the nails can be invaded.

Hyphae and spores may be found in the scales and the appearance is very similar to that of Epidermophyton scales. Primary cultures must be made in liquid media. Growth on maltose agar is scanty and the colony a light gray with a central knob. On glucose agar growth is profuse, the colony is wrinkled and grayish white, changing to amber or brown. The growth consists largely of sterile hyphae and is not characteristic.

Malassezia furfur (*Microsporon furfur, Monilia furfur*): This fungus is the causative agent of *pityriasis (tinea) versicolor,* a disease characterized by a benign macular eruption with scaling and a brownish discoloration of the affected skin. The inflammatory reaction is conspicuously mild. The fungus may be found in large numbers in the scaling epidermis as fragments of branched mycelium and clusters of spores. The latter are thought by some to be conidia arising from the mycelium. In spite of claims to the contrary, it is generally believed that the fungus has not been cultivated and it is, therefore, very poorly known. Both pityriasis versicolor and erythrasma are characterized by recurrences after they have been cleared up by treatment. The organisms seem to be ubiquitous, living on the skin surface of susceptible individuals.

Diagnosis. As indicated earlier, classification of the dermatophytes is based largely on the clinical character of the infection and on colonial morphology on maltose-peptone agar. The differentiated structures developed in culture are, in some instances, of ancillary value. Fermentation reactions, emphasized by Castellani, are generally regarded as of little or no differential value. Animal inoculation is of minor importance unless the lesion is atypical and it is desirable to establish pathogenicity.

The probable cause of the disease can often be closely approximated on the basis of its clinical character; the usual associations are summarized in the accompanying table (p. 689) modified from Henrici.[1] Demonstration of the fungus by direct microscopic examination, however, is essential to establish the etiology and culture is often required for species identification.

The specimen material must be chosen with some care and taken in abundance for both microscopic examination and culture. In ringworm of the scalp epilated stumps of hairs may be taken, and in addition scutula should be taken in favus and the contents of abscesses when the infection is suppurative. In infections of the smooth skin, scrapings from the scaly types should be taken from the margins, rolling toward the normal skin, and the tops of vesicles may be clipped with small scissors in the vesicular types. Macerated epithelium may be taken from intertriginous infections and nail scrapings and subungual hyperkeratotic masses from onychomycosis; the fungous elements are often more difficult to demonstrate microscopically in such specimens.

For direct microscopic examination the material is placed on a slide, a few drops of strong (20 to 40 per cent) sodium hydroxide solution added, covered with a cover glass, and the preparation heated slightly. Only mycelial elements and spores may be found in specimens. Permanent stained preparations may be made by mounting in Amann's medium, a lactic acid-glycerol-phenol solution containing cotton blue. As indicated earlier, sections may be stained by Gram's method or with methyl green-pyronin.

Sabouraud's agar is the medium of choice for primary isolation as well as determination of colonial character. Inoculation should be relatively heavy and a number of plates prepared. Growth is relatively slow, usually ten days to three weeks are required, and the dermatophytes grow well at 30° C. Bacterial contamination may be a source of considerable difficulty, and it must be remembered that contamination with saprophytic fungi from air and dust readily occurs. The method of slide culture has been developed in large part by Henrici[1] and this and other methods of study of the fungi are described fully by him.

III. YEASTS AND YEAST-LIKE FUNGI

Yeasts are often defined as unicellular, nucleated organisms which reproduce by budding. Such a definition is, however, generally recognized as inadequate, in part because some yeasts produce by fission, in part because many, possibly all, produce mycelium under appropriate conditions, and in part because other fungi may exist in a unicellular, yeast-like form which reproduces by budding, *viz.*, the oidia described in the previous section. On the basis of sexual spore formation, some yeasts are ascomycetes, others are probably basidiomycetes, and still others have not been shown to have a sexual stage and are grouped with the Fungi Imperfecti. Clearly, then, the term "yeast" is of somewhat uncertain significance; as usually used it refers to those organisms which exist usually or predominantly in a yeast-like form.[84]
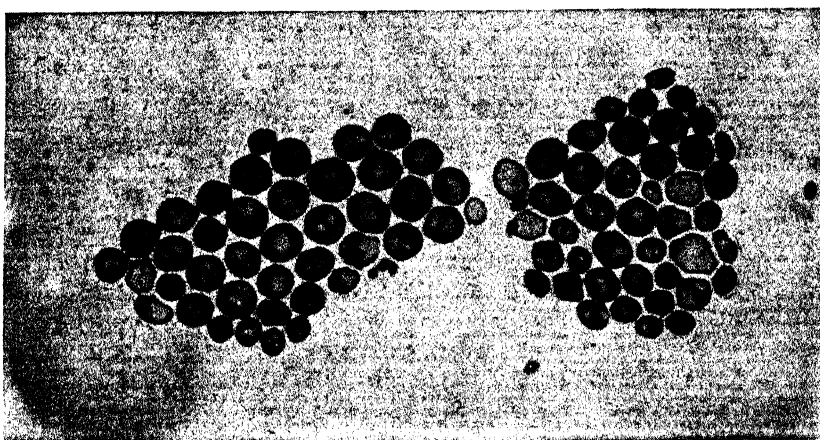
Fig. 181. *Saccharomyces cerevisiae.* Smear from a pure culture. Methylene blue; × 1050.

The yeasts are usually divided into three large groups, the basidiospore-forming yeasts or Sporobolomycetaceae, the ascospore-forming yeasts Endomycetaceae, and the asporogenous yeasts Torulopsidaceae. The industrial yeasts are, perhaps, the most familiar of these. *Saccharomyces cerevisiae,* a member of the second group, is the common brewing yeast and occurs as two types, top yeasts which produce a vigorous evolution of carbon dioxide and are found in the froth on the surface of the fermenting mixture, and the bottom yeasts which sink to the bottom. Bread yeasts are usually top strains of *S. cerevisiae.* Another species of this genus, *Saccharomyces ellipsoideus,* is the common wine yeast, occurring naturally on grapes and in the soil of vineyards, and its varieties are named for the various types of wine which they produce. The mechanism of the alcoholic fermentation has been discussed elsewhere (p. 92). Still other yeasts are lactose-fermenters and are associated with the preparation of fermented milk beverages, such as kefir and koumiss, especially in southeastern Europe. Perhaps the commonest yeasts encountered as contamination in bac-

[84] The genetics, cytology and classification of the yeasts have been reviewed by Henrici: Bact. Rev., 1941, 5:97.

terial cultures and found growing on foods are the asporogenous torulae; the pink or coral pigmented forms often observed are *Torula glutinis.*

In view of the ubiquitous distribution of yeasts, not only in air, dust and soil, but on the surface of the body and in the mouth, intestinal tract and vagina, it is not surprising that these forms have been found in a variety of pathologic processes. A great number of species have been described, most of them inadequately, in this connection. In many instances the yeast probably had no etiologic relation to the disease, and in others the same yeast has been repeatedly described as a new species to give rise to several synonymous names, and a very long list of "pathogenic" yeasts has accumulated.

Critical examination and consideration has now made it clear that only a very few species of yeasts are actually pathogenic for man and animals. These fall into two groups, the one containing the causative organism of European
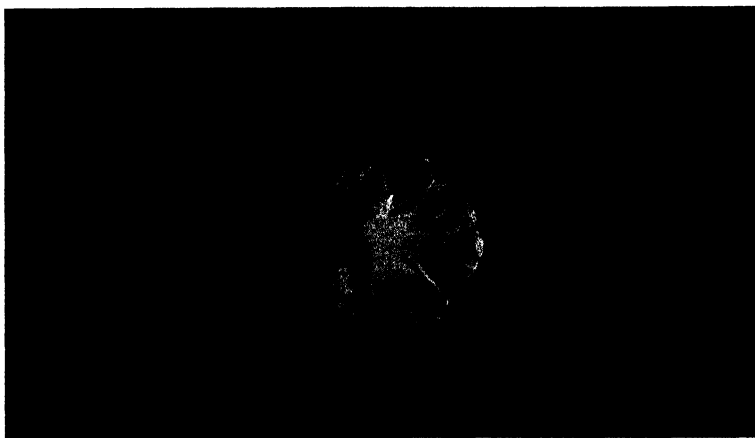
Fig. 182. Giant colony of *Torula glutinis*, a saprophytic species, on Sabouraud's agar; × 2.

blastomycosis or torula meningitis, commonly known as *Cryptococcus hominis* or *Torula histolytica.* As an example of the accumulation of "species" of pathogenic yeasts, Giordano[85] has listed sixty-eight synonyms for this organism. The second group is made up of the "medical Monilias," members of the subfamily Mycotoruloideae of the family Torulopsidaceae, which produce superficial infections of the skin and mucous membranes.

In addition to these a number of yeast-like fungi are considered in this section. These, together with the mold-like fungi discussed earlier, may be regarded as intermediate forms. The differentiation of these forms into "yeast-like molds" and "mold-like yeasts" implied by this separation is largely arbitrary.

CRYPTOCOCCUS HOMINIS (TORULA HISTOLYTICA)

In medical literature the term *blastomycosis* has been used very loosely to designate etiologically diverse infections in which budding cells are found in the tissues. The name itself is unfortunate in that yeasts are not "blastomycetes." Confusion arises largely between European blastomycosis, which will

[85] Giordano: Mycopathologia, 1938–9, *1*:274.

PATHOGENIC YEASTS AND YEAST-LIKE FUNGI

Organism	Disease	Geographic Distribution
Cryptococcus hominis	European blastomycosis, torula meningitis	Europe and U. S., probably ubiquitous
Blastomyces dermatitidis	American blastomycosis	U. S., possibly Europe and elsewhere
Candida albicans	Moniliasis	Ubiquitous
Coccidioides immitis	Coccidioidomycosis	Exclusively southwestern U. S.
Histoplasma capsulatum	Histoplasmosis	Probably ubiquitous, most cases reported from South America and U. S.

be considered here, and American blastomycosis, taken up in the following section.

Aside from moniliasis, two general types of yeast infection may be distinguished, the one the deep-seated cutaneous or subcutaneous infections which tend to become generalized, and the other infections of the central nervous
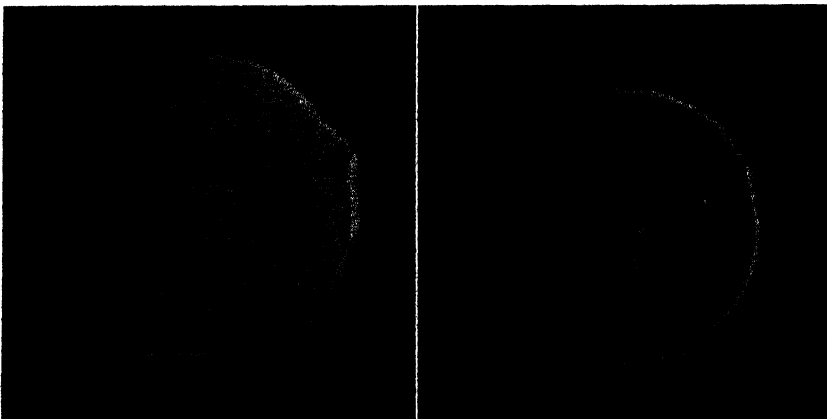
Fig. 183. Giant colonies of torulae isolated from torula meningitis. Strain on left isolated by Shapiro and Neal, on right by Hirsch. Four weeks old, wort agar, natural size.

system arising, as a rule, by metastasis from foci in the lungs. The first of these is known as European blastomycosis and the second as torula meningitis.

European Blastomycosis. What was probably the first case of yeast infection of proven etiology was a fatal generalized infection observed by Busse and by Buschke[86] in 1893 which they called systemic blastomycosis. From primary ulcers on the face and neck the infection spread to the cervical lymph nodes, and the causative organism was isolated first from a secondary tibial

[86] *Cf.* Kolle, Kraus and Uhlenhuth: *Handbuch der pathogenen Mikroorganismen.* Gustav Fischer, Jena. 1928. Vol. 5, p. 321.

abscess, then from the primary ulcers, and shortly before death from the blood stream. Other cases reported since are of this general type, characterized by deep-seated ulceration of the skin, sometimes granulomatous, and there may be infection of the viscera, usually secondary, involving the spleen, liver, kidneys and mesenteric lymphatics. Tumor-like masses are sometimes found and secondary infection of the lungs occurs with some frequency.

The microorganisms are found in exudates and in mucoid masses of gelatinous material as round to oval cells, 5 to 6 μ in diameter, surrounded by a mucilaginous sheath. The gelatinous material in which they may be embedded is evidently a product of the fungus. They are readily cultivated on most ordinary media as a smooth white or very light tan colony without distinguishing features.
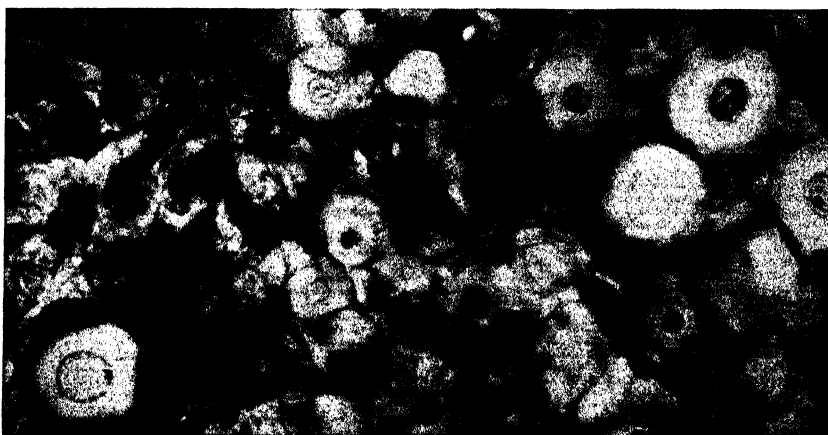
Fig. 184. Torula meningitis in man. Section of the brain showing the growth of *Cryptococcus hominis* in the tissues. Note the yeast-like cells embedded in masses of hyaline material which fill and distort the macrophages. Hematoxylin and eosin; × 950 (Humphreys).

Torula Meningitis. A number of cases of meningitis of yeast etiology have been reported, the great majority from this country. The disease is the subject of a monograph by Stoddard and Cutler[87] who, in 1916, reported two cases and reviewed ten others. In recent years the disease seems to be becoming more common, or at least is reported more frequently. A total of 108 cases were reported to 1946, and there has been only one known recovery from the disease.[88]

The clinical features of the disease set it off sharply from European blastomycosis for the infection is predominantly one of the central nervous system and the skin is seldom involved. The symptoms are those of brain involvement, especially intracranial pressure. Brain tumor may be closely simulated in some cases and the disease develops slowly, usually without febrile reaction

[87] Stoddard and Cutler: *Torula Infection in Man.* Monograph No. 6, Rockefeller Institute for Medical Research. 1916.

[88] *Cf.* Blair: Jour. Mental Sci., 1943, 89:42; Voyles and Beck: Arch. Int. Med., 1946, 77:504.

or other signs of infection. The pathological picture is that of a chronic lepto-
meningitis with thickened meninges adherent to the cerebral cortex and show-
ing diffuse or focal granulomatous lesions. The cerebral cortex is invaded in
about half the cases; the lesions are sometimes granulomatous but more often
cystic and there is little if any inflammatory reaction. The granulomatous lesions
of both meninges and brain contain large accumulations of macrophages which
phagocytose the fungus, while the cystic lesions consist of enormous numbers
of yeast cells embedded in a gelatinous matrix.[89] The yeast is usually present
in the spinal fluid in pure culture and may be observed in wet unstained prep-
arations of the centrifuged sediment. The lung and kidney are the next most
frequently involved but any tissue may be attacked with generalization of the
infection.
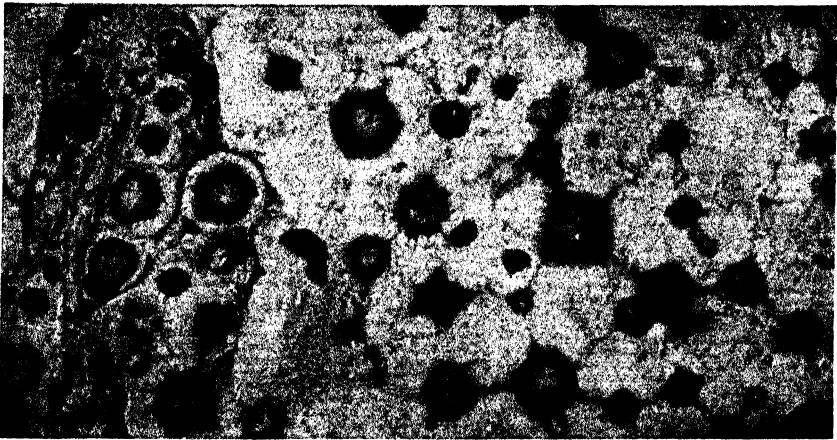
Fig. 185. Torula meningitis in man. Growth of *Cryptococcus hominis* in the cere-
brospinal fluid. Note the budding cells, the stained capsules which give a double-con-
toured appearance, and the characteristic threads connecting the cells. Mucicarmine stain
(staining the threads and capsules pink); × 950 (Humphreys).

The Causative Organism. For some time the relationship of these
clinically diverse infections was not realized, and the yeast of European blasto-
mycosis was known as *Cryptococcus hominis* and that of torula meningitis as
Torula histolytica. In 1934, however, Benham[90] showed that strains of the two
yeasts were substantially identical, the difference in pathogenicity being one
of degree only. Similar observations have been reported by others.[91] It seems
established, therefore, that these diseases are caused by the same etiological
agent and, furthermore, that only a single species of yeast is involved, observed
strain differences having no more than variant status.

Until relatively recently this yeast was regarded as an asporogenous form

[89] The clinical character of the disease and its pathology are discussed in some detail
by Freeman: Jour. Psych. Neurol., 1931, 43:236.
[90] Benham: Arch. Dermat. Syph., 1934, 30:385; Mycologia, 1935, 27:496; Jour.
Inf. Dis., 1935, 57:255.
[91] Lodder: Die Hefesammlung des "Centrallbureau voor Schimmelcultures" II Teil.
Die Anaskosporogenen Hefen. Erster Hälfte. Amsterdam. 1934.

properly grouped under the family Torulopsidaceae. Todd and Herrmann,[92] however, have described the formation of ascospores and their observations have been confirmed and extended to a considerable number of strains by others.[93] Spore formation has some unusual features which need not concern us here, but since single spores are formed by heterogamous conjugation, the organism is referred to the genus Debaryomyces of the family Endomycetaceae. Todd and Hermann[92] suggested the name *Debaryomyces hominis* but Henrici[84] and others have pointed out that the valid name is *Debaryomyces neoformans;* that which continues to be most widely used is *Cryptococcus hominis.*

BLASTOMYCES DERMATITIDIS (AMERICAN BLASTOMYCOSIS)

The fungus disease known as American blastomycosis was first observed in Baltimore in 1894 by Gilchrist[94] and is sometimes called *Gilchrist's disease.*
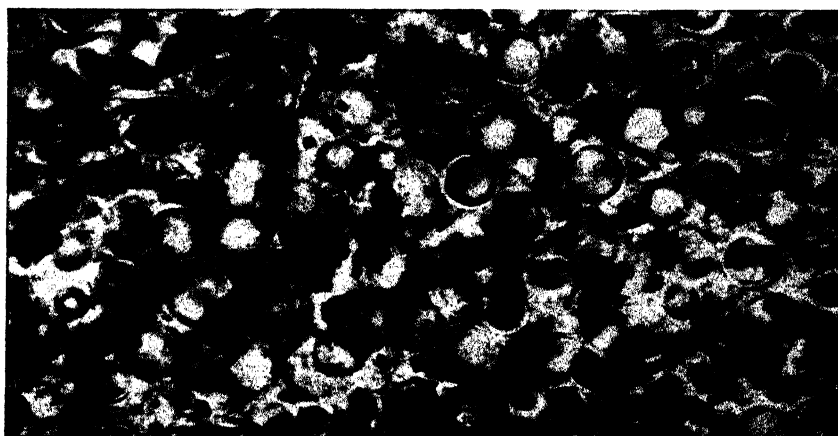
Fig. 186. Blastomycotic pneumonia in man; infection with *Blastomyces dermatitidis.* Note the enormous numbers of yeast cells in the lung tissue and the variation in size and budding forms characteristic of active proliferation. Hematoxylin and eosin; × 1050 (Humphreys).

The great majority of cases have been found in this country, hence the name American blastomycosis, and the disease has been observed more often in the state of Illinois than in other states and is occasionally called *Chicago disease.* According to Martin and Smith[95] only two proved cases have originated outside the United States, one in Canada and one in England, though analysis of presumptive and inadequately described cases is difficult because of the usual failure to distinguish this disease from European blastomycosis.

The Causative Organism. This fungus was isolated by Gilchrist and Stokes[96] from a second case of the disease and named *Blastomyces dermatitidis.*

[92] Todd and Herrmann: Jour. Bact., 1936, 32:89.

[93] Redaelli, Ciferri and Giordano: Boll. Sez. Ital., Soc. Internat. Microbiol., 1937, I-II, 1.

[94] *Cf.* Gilchrist: Johns Hopkins Hosp. Repts., 1896, 1:269.

[95] Martin and Smith: Amer. Rev. Tuberc., 1939, 39:275. This paper is a complete review of the literature to 1939 and a detailed analysis of 347 cases of the disease.

[96] Gilchrist and Stokes: Bull. Johns Hopkins Hosp., 1896, 7:129.

It is dimorphic, occurring only as a unicellular, budding, yeast-like form in the tissues, but as a mycelial form in culture. The unicellular form may be observed in sodium hydroxide preparations of pus or sputum. The cells are large, 8 to 10 μ in diameter, round or oval, and doubly contoured. The granular content of the cells distinguishes them from air bubbles or fat droplets and identification is practically certain if budding cells are found.

Colonial Morphology. This unicellular morphology also occurs in cultures on blood agar, microscopic examination showing budding cells with a few rudimentary hyphae. The colony developing at 37° C. is wrinkled and waxy and somewhat similar to those of the tubercle bacillus in appearance. In culture on Sabouraud's agar the mycelial form appears. The colonial morphology is somewhat variable and Henrici[1] distinguishes three types. The "mealy" type is most often observed in primary isolation cultures and is similar to the growth on blood agar; microscopically the fungus is transitional between the unicellular and mycelial forms with many of the cells tending to form articulated chains. After one or two transfers the growth may assume a prickly surface, the prickles consisting of closely packed mycelial filaments which tend to become loose and cottony with continued incubation. The third type, sometimes observed on primary isolation and commonly found on laboratory cultures which are frequently transferred, is characterized by a white cottony growth with abundant aerial mycelium; in these colonies the unicellular form has completely disappeared and the growth consists of septate hyphae. Conidia are borne on lateral conidiophores but reports by some workers of the formation of asci have not been generally confirmed. The mycelial form reverts to the unicellular type in experimental infections or when cultivated on blood agar.

Classification. The relationships of this fungus are somewhat uncertain though it is clearly not a "true yeast." Taxonomic considerations have led to the proposal of a number of different names; these include *Oidium dermatitidis, Gilchristia dermatitidis, Cryptococcus gilchristi, Mycoderma dermatitidis, Zymonema dermatitidis* and others. Systematists object to all these for one reason or another and none has been generally accepted. In spite of the fact that, strictly speaking, the generic name Blastomyces is improper from the taxonomic point of view, it continues to be generally used and the fungus is known as *Blastomyces dermatitidis.*

Pathogenicity. Primary infection occurs most commonly in the skin and the lesions begin as a small firm papule about which secondary nodules develop which enlarge with coalescence. The process breaks down in the center and becomes suppurative with discharge of pus through small fistulae. The inflammatory reaction is granulomatous with connective tissue formation, proliferation of the epithelium, mononuclear infiltration and sometimes giant cell formation. The fungus is found in the pus of minute miliary abscesses which are a characteristic feature of the pathology. As the disease progresses, therefore, a large elevated mass of tissue, with an irregular ulcerated surface, is formed and oozes pus from multiple small openings upon pressure. The resemblance to epithelioma or tuberculous ulcer is sometimes striking. Lesions with pronounced epithelial proliferation simulate tuberculosis verrucosa. The process spreads slowly through the subcutaneous tissues and often becomes generalized by way of the bloodstream.

Primary infection of the lungs is not uncommon and often closely resembles

tuberculosis clinically with cough, pain in the chest, weakness, sometimes hemoptysis, and productive sputum late in the disease. The pathologic picture is variable in that there may be focal or diffuse consolidation, and the abscesses may be miliary or there may be larger nodules. Cavitation occurs but is limited to small areas. The microscopic picture also resembles tuberculosis and sometimes it is difficult to differentiate unless the budding cells are found.

With generalization from primary foci of infection, multiple small abscesses of hematogenous origin occur throughout the body. They are most common in the subcutaneous tissues and differ from the primary skin lesions in that they develop without pain or marked erythema, are soft and evacuate considerable quantities of pus when opened. In contrast to the primary skin lesions, which most commonly occur on exposed parts of the body, the secondary subcutaneous abscesses are usually found in covered areas. Secondary abscesses also commonly develop in the viscera and muscles and under the periosteum. There is a septic febrile reaction and the generalized disease is probably almost always fatal.[97]

The source of infection is not clear. Very often the primary skin lesion develops from a wound infection and a large proportion of the cases have been in farmers. Males are much more frequently attacked and the incidence is higher in the twenty to fifty age group. It is possible that the fungus is a free-living form which, like Sporotrichum, occasionally infects man. One case of direct transmission, by autopsy infection, is known.

Pathogenicity for Animals. The inoculation of experimental animals is not uniformly successful and has no diagnostic utility. According to Spring[98] mice are more susceptible than guinea pigs, and rabbits are almost completely resistant. Small caseous nodules develop on the peritoneal surfaces of intraperitoneally inoculated animals, and the type of tissue reaction varies with the resistance of the animal and the virulence of the strain of fungus from frank abscess formation to tubercle-like lesions. Generalized blastomycosis has been demonstrated in a naturally infected dog and the fungus found to be identical with typical strains of human origin.[99]

Immunity. There appears to be little or no effective immunity in man to spread of the infection since generalization is common. Complement-fixing antibodies are produced, however, and the titer is related to the severity of the infection. A hypersensitivity to the cell substance of the fungus occurs also and is manifested as a delayed tuberculin-like response to intradermal inoculation of suspensions of killed cells. The hypersensitivity is of practical importance in that it contraindicates the therapeutic use of iodides; desensitization may be accomplished with vaccine. It has not as yet been possible to demonstrate an effective immunity to the experimental infection in laboratory animals.

Diagnosis.[100] As indicated above, the yeast-like unicellular form of the fungus can be demonstrated by direct microscopic examination of pus or sputum mounted in sodium hydroxide. Positive complement-fixation and skin

[97] The histopathology of blastomycosis is discussed by Baker: Amer. Jour. Path., 1942, 18:479.

[98] Spring: Jour. Inf. Dis., 1929, 44:169.

[99] Foshay and Madden: Amer. Jour. Trop. Med., 1942, 22:565.

[100] For a detailed discussion of the usual methods of laboratory diagnosis see Martin: Jour. Inf. Dis., 1935, 57:291; Martin and Smith: Jour. Lab. Clin. Med., 1936, 21:1289.

tests are of value in pulmonary and systemic infections. An unequivocal diagnosis can be established, however, only by isolation and identification of *Blastomyces dermatitidis*. This is usually not difficult if the specimen is not too heavily contaminated with bacteria; the fungus grows readily on Sabouraud's agar, the colonial morphology varying as indicated earlier.

CANDIDA ALBICANS (MONILIA ALBICANS)

The fungi which makes up the group of "medical monilias" are yeast-like organisms which are usually found in the budding unicellular stage. Mycelium is formed only under semi-anaerobic conditions or in submerged cultures, and may be found in the tissues. When cultured on agar in the usual way, the colonies are smooth and waxy and on microscopic examination are found to consist entirely of budding, yeast-like cells. Gelatin stab culture is a convenient
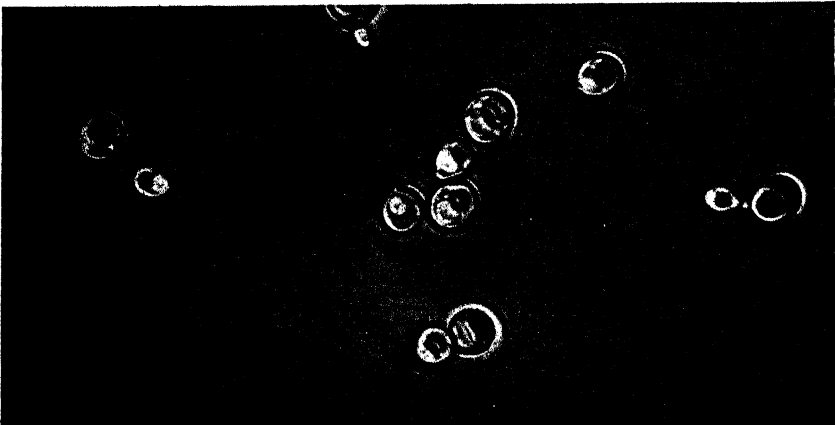
Fig. 187. Unstained preparation of the yeast-like form of *Candida albicans*. Oblique lighting. Note the budding cell and close resemblance to the true yeasts. × 1050 (Rothman).

method of differentiating these forms from the yeasts for, while the surface growth is yeast-like, growth along the line of inoculation includes the extension of radiating filaments into the medium; these consist of mycelium from which budding cells arise. The same type of growth may be observed on agar plates by cutting deeply into the medium with the inoculating needle and examining the growth through the bottom of the plate with the low power. In general, under conditions favorable for growth and rapid multiplication, the yeast-like form predominates, while mycelium appears under less favorable conditions.

The first description of a fungus of this type occurring in disease was that of Langenbeck in 1839 who found it in patches on the mucous membranes of the mouth and elsewhere at autopsy. The organism was named *Oidium albicans* by Robin four years later. The generic name Monilia has come to be commonly used and the disease is usually termed *moniliasis*. The moniliases are usually superficial infections of the mucous membranes, the intertriginous areas and the nails, but pulmonary moniliasis is known and localized infections some-

times result in a generalized infection by hematogenous spread, especially in hosts debilitated by some other disease.

The Causative Organism. In addition to the original *Monilia albicans*, a considerable number of species of Monilia have been described including *Monilia krusei, Monilia parakrusei, Monilia tropicalis, Monilia stellatoides* and others. In fact, a large proportion of current literature on these organisms is devoted to their differentiation, identification and classification. Differentiation is made on the basis of various cultural characters such as pellicle formation in liquid media, gelatin liquefaction and the like, and Castellani[101] has especially emphasized differential fermentations. The last is the most practical laboratory method but there is some disagreement as to the constancy of these characteristics. The various strains do not make up an immunologically homogeneous group but are closely related and show pronounced cross reactions.[102] It is
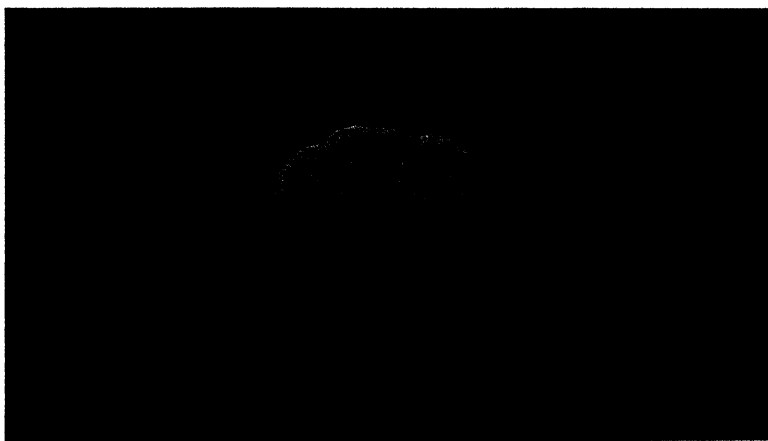
Fig. 188. Giant colony of *Candida albicans* on Sabouraud's agar. Note the smooth creamy growth characteristic of yeasts. × 3.

generally admitted, however, that there is no marked correlation of the various differential characters, and that species so differentiated are not associated with any particular type of infection, *i.e.*, the same species may cause a variety of disease processes and different species are found in the same sort of lesion. A further complicating factor is the inherent variability of these fungi; smooth, rough and transitional forms are observed, sometimes occurring simultaneously in the same patient. While the question of the plurality of species is by no means closed, the differentiation of a considerable number of species appears to be of limited value and justified only in part.[103] For the present, therefore, we shall assume that all the pathogenic monilias constitute a group of varieties of the single species, *Monilia albicans*.

Considerable interest has attached also to the proper generic name for these

[101] *Cf.* Castellani: Jour. Trop. Med. Hyg., 1937, *40*:293.

[102] Martin: Amer. Jour. Trop. Med., 1942, 22:295.

[103] See Langeron and Guerra: Ann. Parasitol., 1938, *16*:36, 162, 429, 481. These authors review the literature critically, give a detailed discussion of the methods of differentiation, and recognize seven species of the genus.

fungi. As indicated above, Oidium was used very early. A number of old
generic names have never been widely used. It is generally recognized that
Monilia, commonly used in medical literature, is a misnomer, for these yeast-like
fungi are quite different from those included by the mycologist in the genus
Monilia Persoon 1797. The name Mycotorula has been suggested in recent
years, but the name Candida is probably preferable and in any case is coming
into common use. The organism is, therefore, *Candida albicans* and, if other
species are differentiated, they are properly species of Candida.

Pathogenicity. These fungi are commonly present in the mouth, vagina
and intestinal tract in normal persons and are probably not highly virulent
with respect to initiation of the infectious process. In fact, as in many of the
mycoses, there is frequently a history of a debilitated condition or other pre-
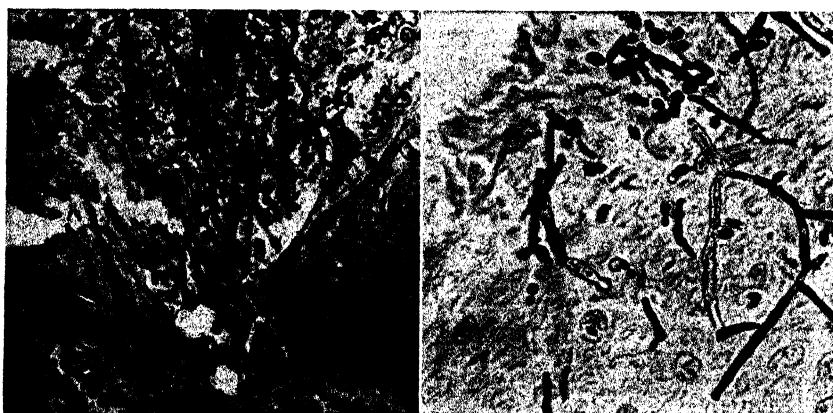
Fig. 189. Moniliasis: invasion of the epithelial layer of the esophagus by *Candida
albicans*. Left, beginning invasion of the superficial layer showing mycelium and yeast-
like cells; note the gram-positive micrococci accompanying the fungus; × 600. Right,
invasion of the deeper tissue showing both mycelium and yeast-like cells penetrating the
epithelium; × 950. Gram stains (Humphreys).

disposing factors. While in some instances in which they are associated with
the development of a pathologic process there is some doubt as to their etiologic
role, in many others it is clearly evident that they are primary invaders and re-
sponsible etiologic agents.

Infections of the Mucous Membranes. Moniliasis of the mucous membranes
is known as *thrush* and is one of the common mycoses. Intra-oral thrush, a
superficial infection of the mucous membranes of the mouth, is the type most
frequently observed and is considerably more common in nursing infants and
children than in adults. In the past it has occurred in severe epidemic form in
institutions such as foundling asylums but such epidemics are observed less
often now. Henderson[104] has reported an incidence of 6.3 per cent in infants
in the Simpson Maternity Hospital, Edinburgh, in 1940; strict isolation pro-
cedures reduced this to 3.7 per cent in 1942. Thrush occurs more frequently
in bottle-fed than in breast-fed babies, and the lesions not infrequently spread

[104] Henderson: Edinburgh Med. Jour., 1943, 50:535.

to the pharynx and even the esophagus. In adults thrush generally occurs terminal to wasting diseases such as tuberculosis and cancer; dryness of the mouth associated with prolonged coma or near coma appears to favor infection. It also occurs with some frequency as a mild vaginal infection in pregnant women which may or may not be associated with infection of the anus.

In the majority of cases the infection is mild and remains localized. Occasionally it may spread to other mucous membranes and to the skin with the development of a generalized cutaneous eruption and intertriginous lesions, and such cases are sometimes fatal. The most common predisposing malady for cutaneous moniliasis is diabetes. Hematogenous spread with metastasis and abscess formation in the viscera has been observed.

The lesions appear as soft, whitish patches, composed largely of fungous growth. They are readily removed to leave an eroded surface. There is some resemblance to a diphtheritic membrane and lesions in the throat and on the tonsils have no doubt been mistaken for diphtheria. However, it is much easier to separate the membrane in moniliasis. On microscopic examination of the membranous material in wet preparation, the observation of a tangled mass of segmented mycelium admixed with budding yeast-like cells, desquamated epithelium and leucocytes establishes the diagnosis. The fungus may be cultivated on Sabouraud's agar but isolation in pure culture is more readily accomplished with dextrose tartaric acid agar. The isolated fungus must be differentiated from yeasts.

Dermatomoniliasis. C. *albicans* is also causally associated with eczema-like lesions of the moist skin similar to those produced by *Epidermophyton floccosum*. Infection of the folds between the fingers, erosic *interdigitalis*, is more common than dermatophytosis of this region, and is found most ften in those whose hands are frequently wet. *Perlèche*, an infection of the ngles of the mouth, is another form of intertrigo produced by these fungi. *Moniliids*, sterile vesicular or exudative lesions which appear on the hands secondary to a focus of infection elsewhere, are analogous to trichophytids and a result of hypersensitivity.

The nails, chiefly of the fingers, are also attacked by C. *albicans* with the production of chronic paronychia. The condition is differentiated clinically from onychomycosis by retention of the luster of the infected nail and the absence of yellowish discoloration, crumbling and thickening of the soft tissues. The nail shows transverse ridges and eventually becomes thickened, distorted and brownish in color. Like interdigital infections, the infection is found most often in those whose hands are often wet.

On microscopic examination of nail and skin scrapings cleared in 10 per cent sodium hydroxide, hyphae may be present together with the budding yeast-like cells.

Bronchomoniliasis. There appears to be a distinct type of moniliasis of the respiratory tract which was first reported by Castellani[105] from Ceylon. It has been observed in Europe and this country but is apparently more common in the tropics. A number of moniliae have been isolated, all closely related, but that variety known as *Candida tropicalis* has been most often found. According to Castellani, two types of the disease occur; the mild form, a chronic bron-

[105] Castellani: Lancet, 1922, i:13; Military Surgeon, 1925, 57:113.

chitis characterized by dyspnea and cough, is afebrile, while the severe form is similar to tuberculosis and usually fatal.

The fungi may be demonstrated by direct microscopic examination and by culture but such findings must be interpreted with caution, for the organism is frequently found in the sputum in other diseases, especially tuberculosis. It is recommended that the throat be cleansed by gargling before the sputum is collected and other contamination avoided. Strains isolated from cases of bronchomoniliasis are pathogenic for rabbits, producing tubercle-like nodules within two or three weeks after inoculation into the lungs.

COCCIDIOIDES IMMITIS

Coccidiomycosis, coccidioidomycosis or coccidioidal granuloma was first observed in Argentina in 1892 by Posados[106] and by Wernicke[107]; two years later it was described independently in California by Rixford.[108] The causative

Fig. 190. Colony of *Coccidioides immitis* on blood agar. Isolated from a fatal case of coccidioidomycosis. Natural size.

organism was thought to be a protozoan and named *Coccidioides immitis*. Ophuls and Moffitt[109] showed, by culture, that it is a fungus but this does not invalidate the name. Though originally reported from South America, the disease appears to be rare there and is most prevalent in the San Joaquin Valley in California and in the dry regions of the southwestern United States; it seems to be unknown elsewhere.

The Causative Organism. Like a number of the other pathogenic fungi, *C. immitis* is dimorphic, and the parasitic stage within the tissues is morphologically different from that assumed in culture.

The Parasitic Stage. This fungus differs markedly from the other yeast-like fungi in that it never reproduces by budding, and it differs from all the other pathogenic fungi in that it reproduces within the tissues exclusively by a

106 Posados: Ann. Circ. Med. Argentina, 1892, 15:585.
107 Wernicke: Cenntralbl. f. Bakt., 1892, 12:859.
108 Cf. Rixford and Gilchrist: Johns Hopkins Hosp. Repts., 1896, 1:209, 253.
109 Ophuls and Moffit: Philadelphia Med. Jour., 1900, 5:1471.

process of endogenous spore formation. The newly liberated spores are small mononucleate spheres 1 to 3 μ in diameter and appear as a central, deeply stained mass of protoplasm surrounded by a double-contoured capsule. The cell enlarges, soon becoming multinucleate, and eventually reaches a diameter of 50 to 60 μ. A central vacuole appears early and in later stages occupies a large part of the cell, the protoplasm appearing as a thin peripheral layer. The peripheral protoplasm becomes vacuolated and an indefinite number of multinucleate protospores become delimited by the formation of cell walls; the protospores in turn subdivide to form spores and the entire cell assumes the functions of a sporangium. With rupture of the cell wall the mature spores are liberated and the developmental cycle begins again.[110] All of these forms may
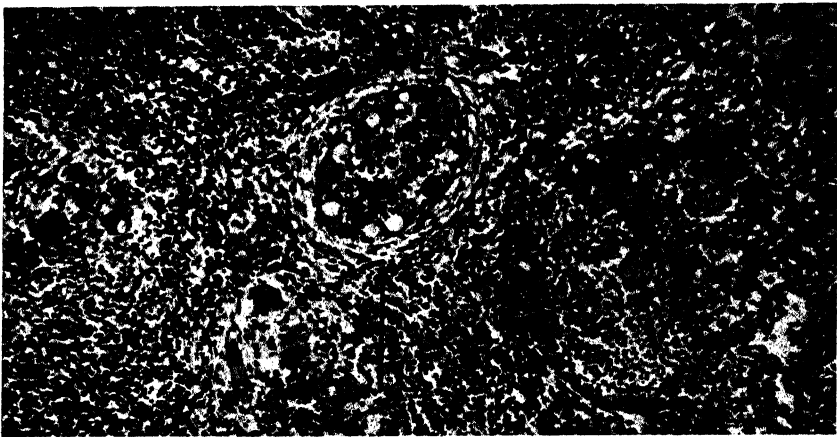
Fig. 191. Coccidioidomycosis in man. Section of spleen showing a large lesion of the chronic type and two smaller lesions characteristic of the acute type of infection. In the latter sporulation is complete and the spores are beginning to spread into the surrounding tissue. Hematoxylin and eosin; $\times$ 125 (Humphreys).

be observed in the tissues and in pus, though recently disseminated spores are difficult to demonstrate.

The Saprophytic Stage. The fungus will grow on a variety of sugar-containing media but Sabouraud's agar is the medium of choice. It grows best at 37° C. under aerobic conditions. On artificial culture media the growth is that of a mold. The colony may be smooth and waxy when young but aerial mycelium is soon formed and it becomes gray or brownish in color. Emmons[110] has pointed out that, though it is often stated that the aerial hyphae break up into chlamydospores, the spores are borne on differentiated conidiosphores and should be termed conidia or oidia. Spirals are frequently observed.

Pathogenicity. For many years infection with *C. immitis* was known only as coccidioidal granuloma, a severe disease in which the case fatality rate was thought to be 90 per cent or more. Infection with this fungus is, however,

[110] Nuclei were first described by Emmons in a recent review which includes complete descriptions of both parasitic and saprophytic stages of the organism as well as a discussion of the disease. *Cf.* Emmons: Mycologia, 1942, 34:452.

much more prevalent and less severe than had been supposed. Dickson[111] has observed healed lesions of coccidioidal granuloma at autopsy of persons dying of other causes, and Cox and Smith[112] have reported similar findings and produced arrested lesions in white rats. Furthermore, it was shown by Dickson[113] in 1937 that the disease may take a second form, a benign, acute, self-limited respiratory infection, in addition to the chronic, progressive granulomatous disease. He suggested that these be designated primary and secondary or progressive coccidioidomycosis respectively and these terms are now coming into general use.
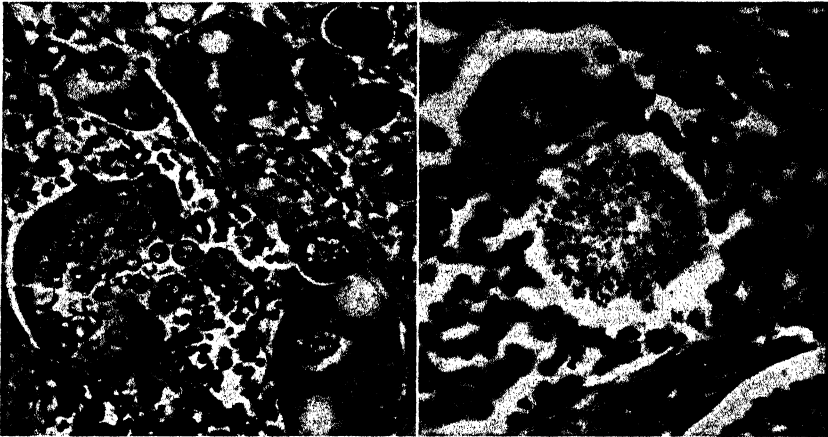
Fig. 192. The chronic and acute types of lesion in coccidioidomycosis in the same section shown in Fig. 191, but under higher magnification. Left, the chronic type of lesion showing many mature cells of *Coccidioides immitis*: × 475. Right, the active type of lesion showing a mass of spores beginning to spread in the tissue. Hematoxylin and eosin; × 710 (Humphreys).

Primary Coccidioidomycosis. The infection is acquired by inhalation of the spores, and the disease varies in severity in recognized cases from that of a common cold to cases resembling influenza with pneumonia, cavitation and high fever. In a small proportion of the cases, possibly 5 per cent, an exanthem like erythema nodosum occurs and the disease is recognized as "the bumps," desert rheumatism, valley fever, San Joaquin fever, etc. Many infections are symptomless, however. In a group of 1351 cases studied by Smith *et al.*[114] 60 per cent were symptomless and dissemination with development of the progressive disease occurred in about 1 per cent of the clinically apparent infections. They also noted that dissemination occurred ten times as frequently in the Negro as in the white. Minor "epidemics" may occur such as that described by Davis, Smith and Smith[115] in which seven of fourteen persons who

[111] Dickson: Arch. Int. Med., 1915, *16*:1028.
[112] Cox and Smith: Arch. Path., 1939, 27:717.
[113] Dickson: Calif. & Western Med., 1937, 47:151. See also Arch. Int. Med., 1937, *59*:1029; Amer. Rev. Tuberc., 1938, 38:722; Jour. Amer. Med. Assn., 1938, *111*:1362.
[114] Smith, Beard, Whiting and Rosenberger: Amer. Jour. Pub. Health, 1946, 36:1394.
[115] Davis, Smith and Smith: Jour. Amer. Med. Assn., 1942, *118*:1182.

spent two days on a desert field trip near the San Joaquin Valley became infected, and Goldstein and Louie[116] reported that of several thousand soldiers exposed during military training in a desert endemic area, seventy-five contracted the disease. All but one made rapid progress to complete recovery, but the remaining individual developed a generalized infection and coccidioidal granuloma. Spontaneous recovery is the rule and only rarely does the disease progress to the secondary type.

Secondary or Progressive Coccidioidomycosis. The progressive type of infection results in cutaneous, subcutaneous, visceral and osseous lesions. The cutaneous type closely resembles blastomycosis but is a much more severe disease with fever and a greater tendency to hematogeous spread. Elsewhere the lesions closely resemble those of tuberculosis and, in fact, differentiation from that disease may often be possible only by demonstration of the fungus.[117] It is not known whether the progressive type of disease results from a new infection or reactivation of arrested primary lesions.

Pathogenicity for Animals. The disease occurs naturally in domestic animals including cattle, sheep and dogs and also in certain wild rodents[118] in endemic areas. The latter include three species of pocket mice, *Perognathus baileyi, P. penicillatus* and *P. intermedius,* the kangaroo rat, *Dipodomys merriami,* and the grasshopper mouse, *Onychomys torridus.* Experimental animals are readily infected; both rabbits and guinea pigs are susceptible to intraperitoneal inoculation.

Immunity. Since it is now apparent that infection with *C. immitis* can take a mild form from which spontaneous recovery is the rule, that arrested lesions occur with some frequency, and that the case fatality rates in the severe progressive type of disease are not as high as once thought, it is indicated that there is, on the one hand, an appreciable natural resistance to infection, and on the other, an effective immune response.

The immune response is manifested in part as the development of a hypersensitivity to the parasite. *Coccidioidin* is, like tuberculin (OT), prepared from liquid cultures of the parasite, but there is some difficulty in reproducing potencies. Hassid, Baker and McCready[119] have described an immunologically active polysaccharide which appears to make up at least a part of the active principle, producing both skin reactions and precipitin test. Coccidioidin is inoculated intradermally, a positive reaction appearing in twenty-four to forty-eight hours. The hypersensitivity appears in a few days to a few weeks after infection, recent infections and severe infections resulting in stronger reactions than old infections and mild infections respectively. The test appears to be highly specific though there is as yet some disagreement as to its interpretation.[120]

Diagnosis. The laboratory diagnosis of coccidioidomycosis is dependent upon the demonstration of the parasite, the diagnostic value of a positive coccidioidin reaction being somewhat uncertain. As indicated earlier, it may

[116] Goldstein and Louie: War. Med., 1943, 4:299.
[117] See Clark and Gilmore: Ann. Int. Med., 1946, 24:40.
[118] *Cf.* Emmons and Ashburn: Pub. Health Repts., 1942, 57:1715.
[119] Hassid, Baker and McCready: Jour. Biol. Chem., 1943, 149:303.
[120] *Cf.* Stewart and Kimura: Jour. Inf. Dis., 1940, 66:212.

be found on direct microscopic examination of pus, spinal fluid and tissues, but may be difficult to demonstrate in the sputum. Lactophenol-cotton blue or Mallory's eosin and methylene blue may be used for staining. Though *C. immitis* grows without difficulty on sugar-containing media, it has been cultured in a surprisingly small proportion of cases; in 432 cases studied by Smith,[121] the fungus was recovered from only 22 per cent of the patients.

Epidemiology. In the endemic areas of the southwestern United States the proportion of reactors to the coccidioidin test is high, ranging from 16 to 90 per cent.[122] The prevalence of infection in these regions appears, therefore, to be much higher than suspected earlier. A very large proportion of these reactors have no history of coccidioidomycosis—Smith[121] estimated that only 5 per cent of infected persons show sufficiently pronounced clinical symptoms to be detected and diagnosed.

Though, as indicated earlier, the disease occurs in domestic animals and certain wild rodents, there is no evidence of direct transmission of the infection from animals to man. It seems likely both man and animals are infected by the inhalation of spores contained in dust; there is frequently a history of exposure to dust storms in human cases and the fungus has been isolated directly from dust.[123] *C. immitis* is probably not, however, a saprophyte which infects animals and man by chance, but rather a parasite, the reservoir of infection of which appears to be wild rodents that discharge spores in the feces and thus contaminate the soil, resulting in transmission of the fungus to both other wild rodents and man.[124]

Paracoccidioidomycosis. The disease known as paracoccidioidal granuloma or South American blastomycosis occurs in Brazil but has not been observed in this country. The causative organism is *Paracoccidioides brasiliensis* which, despite its name, is not closely related to *C. immitis*. It is a yeast-like fungus, proliferating by budding in the tissues, and grows in a compact cerebriform colony on Sabouraud's agar, eventually producing a white aerial mycelium.

The disease is clinically similar to coccidioidomycosis and blastomycosis. The fungus enters the body via the mouth to produce ulcerative granulomatous lesions which spread to the tongue, lips and nose. The infection spreads via the lymphatics to produce lesions of the viscera and often pulmonary involvement. It is usually fatal.

Diagnosis is established by microscopic demonstration of the multiple budding cells (in contrast to the singly budding cells of *Blastomyces dermatitidis*) in material from the lesions, or by culture.

HISTOPLASMA CAPSULATUM

This organism was discovered in 1906 by Darling[125] who observed it in sections of tissues taken post mortem from three cases of what appeared to be visceral leishmaniasis and named it *Histoplasma capsulatum*. The first case in

[121] Smith: Amer. Jour. Pub. Health., 1940, 30:600.
[122] Farness: Jour. Amer. Med. Assn., 1941, 116:1749.
[123] *Cf.* Emmons: Pub. Health Repts., 1942, 57:109.
[124] *Ibid.*, 1943, 58:1.
[125] Darling: Jour. Amer. Med. Assn., 1906, 46:1283.

the United States was reported in 1926. The disease is reviewed by Meleney[126] who summarized 32 cases known to 1940, all of which were American and 24 of which were found in the United States. Since then other cases have been reported and Parsons and Zarafonetis[127] have reviewed a total of 71 cases reported to 1945. The increasing number of cases reported in recent years is no doubt a consequence of more general interest in the disease and suggests that it is more common than has been thought.

The Causative Organism. The organism appears in the tissues as a small, oval, encapsulated yeast-like cell 3 to 5 μ in diameter. Larger strains, 10 μ in diameter, have been found in two cases and described as another species, *Histoplasma pyriforme*, but the implied difference or relationship is highly un-

Fig. 193. *Histoplasma capsulatum.* Yeast-like cells in the macrophages of the bone marrow in systemic histoplasmosis. Hematoxylin and eosin; × 1000 (Humphreys).

certain. In stained preparations the central stained mass is surrounded by a clear zone and the refractile capsule. The organism is usually intracellular, in the mononuclears in the peripheral blood and in the macrophages elsewhere, especially those of the bone marrow and spleen. Because of this characteristic intracellular position of the parasite, the disease is sometimes called *reticuloendothelial cytomycosis* or simply *cytomycosis*.

The organism was cultivated in 1934 by DeMonbreun[128] from the blood during life and the spleen at autopsy of one case, and by Hansmann and Schenken[129] from biopsy material from another. It grows in the yeast-like form seen in the tissues in sealed blood agar cultures, but on Sabouraud's agar a mycelial form is assumed and the colonies are mold-like, white and cottony with aerial mycelium. Chlamydospores are formed in abundance, first smooth and pyriform, but as they mature they become larger, 7 to 15 μ in length, thick-walled and tuberculate with finger-like protuberances sometimes as much as 6 μ long. Conant[130] has studied these developmental stages in detail and has

126 Meleney: Amer. Jour. Trop. Med., 1940, 20:603.
127 Parsons and Zarafonetis: Arch. Int. Med., 1945, 75:1.
128 DeMonbreun: Amer. Jour. Trop. Med., 1934, 14:93.
129 Hansmann and Schenken: Amer. Jour. Path., 1934, 10:731.
130 Conant: Jour. Bact., 1941, 41:563.

been able to convert the mycelial stage to the yeast-like form by cultural methods. The fungus has been regarded by some as closely related to *Para-coccidioides brasiliensis*.

Pathogenicity. Meleney[126] has differentiated four general types of disease produced by *H. capsulatum*. The clinical picture is sometimes one of a systemic febrile disease with splenomegaly, septic temperature curve, anemia and leucopenia, simulating kala-azar. A second type is distinguished by the predominance of lymphatic involvement and resembles Hodgkin's disease, lymphosarcoma, leukemia or aplastic anemia. Or pulmonary symptoms may predominate with cavitation and fibrous adhesions of the pleurae, the infection sometimes complicating tuberculosis; the development of calcified intrathoracic nodules is perhaps a consequence of pulmonary infection. A fourth type is characterized by ulcerative skin lesions; ulceration also occurs in the intestine.

Fig. 194. *Histoplasma capsulatum*. Yeast-like cells in the suprarenal gland in systemic histoplasmosis. Gram stain; × 1350 (Humphreys).

Though it is sometimes implied that histoplasmosis is always a generalized infection of the macrophage system, the parasites being especially numerous in tissues rich in these cells such as the spleen and bone marrow, this is by no means generally true. In most cases there are nodules or extensive areas of necrosis in one or more organs and in some instances only a single organ, such as the adrenals, has been infected. These necrotic lesions usually consist of a central area of necrosis surrounded by granulation tissue containing large numbers of macrophages and ingested parasites. In some instances the parasite has been found to be limited to such areas, while in others it is widely distributed in the macrophage system as well. In any case the prognosis is bad, for the disease is almost invariably fatal (however, see below).

Experimentally infected animals give a positive reaction to the intradermal inoculation of a preparation of liquid culture of *H. capsulatum* analogous to old tuberculin and coccidioidin and designated *histoplasmin*. There is a considerable degree of cross reaction with Blastomyces and the specificity of the histoplasmin reaction is not altogether clear.[181] It has been of particular inter-

181 Emmons, Olson and Eldridge: Pub. Health Repts., 1945, 60:1383.

est, however, since Palmer[132] has reported finding a high percentage of positive reactors in areas from which the majority of cases have been reported, roughly the central-eastern part of the United States, which also coincides with the geographical distribution of occurrence of pulmonary calcification of non-tuberculous origin. The percentage of positive reactors was 61.5 in Kansas City, Missouri, and 50.2 in Kansas City, Kansas, in contrast with 4.7 in Minneapolis. Similar results were reported in Tennessee by Christie and Peterson,[133] and Palmer[134] further found that within an area comprising Tennessee, Kentucky, Arkansas, Missouri, Indiana and parts of Ohio, Illinois, Kansas and Louisiana, positive reactions averaged 68.3 per cent, varying within it from 78.8 per cent in northwestern Missouri and northeastern Kansas to 6.9 per cent in the northwestern corner of Kansas. In sharp contrast the areas from the Great Lakes to the Pacific coast and from Colorado to the Canadian border showed only 1.4 per cent reactors. Within Kansas City, Furcolow, High and Allen[135] found that the proportion of positive reactors increased from 5 per cent at the age of two years to 60 per cent at eighteen years of age, and that 90 per cent of males and 75 per cent of females over age fifty-five gave positive reactions, all positively correlated with the incidence of pulmonary calcification in tuberculin-negative individuals. The association of positive histoplasmin reactions with calcified nodules in the lungs suggests that, as in the case of coccidioidomycosis, histoplasmosis is not a rare, highly fatal disease, but one which is widespread in certain geographical areas in a mild pulmonary form. As yet, however, this is no more than an inference for direct evidence is lacking. In only one instance was *H. capsulatum* isolated from a hilar lymph node (Christie and Peterson) and that from an infant that did not die of histoplasmosis; other cultures of pulmonary calcifications have been negative as were those of sputum from an epidemic of atypical pulmonary disease in Tennessee. In the opinion of Groover[136] the histoplasmin test is of but limited value in the differential diagnosis of pulmonary disease.

Pathogenicity for Animals. A naturally occurring infection in a dog has been described by DeMonbreun[137] and was experimentally transmitted to puppies by oral and parenteral inoculation. Infections of dogs have since been found by others also. Similar infections of ferrets and mice have been reported though not proved to be histoplasmosis. Experimental animals are infected with some facility, a localized lesion being produced at the site of subcutaneous inoculation in guinea pigs and rabbits, and generalized infection in dogs and rats.[129] Both yeast-like and mycelial forms are infectious experimentally.

Diagnosis. Microscopic demonstration of the fungus is highly suggestive but culture and identification are required to establish the diagnosis. *H. capsulatum* has been isolated by blood culture in generalized infections and from biopsy specimens; sternal puncture may prove useful. In any case, the mycelial form of the fungus developing on Sabouraud's agar at room temperature is distinguished from other pathogenic fungi by the formation of the large, thick-walled, tuberculate chlamydospores described above.

[132] Palmer: Pub. Health Repts., 1945, 60:513.
[133] Christie and Peterson: Jour. Amer. Med. Assn., 1946, 131:658.
[134] Palmer: Pub. Health Repts., 1946, 61:475.
[135] Furcolow, High and Allen: Pub. Health Repts., 1946, 61:1132.
[136] Groover: Arch. Int. Med., 1947, 80:496.
[137] DeMonbreun: Amer. Jour. Trop. Med., 1939, 19:565.

THE SPIROCHETES

Spirally shaped microorganisms were for a long time grouped together indifferently under the common name of spirillium, spirochete or vibrio. Further knowledge has shown that some of these are true bacteria, although curved, and such forms are now placed with the bacteria in the genus Vibrio and various species of Spirillum. Others, however, possess characteristics which distinguish them from curved bacteria, such as the cholera vibrio, and also from the actively motile protozoa, such as the trypanosomes. These forms, the spirochetes, are actively motile but, unlike the motile bacteria, not by means of flagella but rather through the rotation of the screw-shaped cell. Some spirochetes possess a terminal filament which functions as an organ of locomotion and resembles the polar flagellum of monotrichous bacteria. Like the bacteria, however, the spirochetes multiply by transverse fission, have no well-defined nucleus, and do not exhibit anterior-posterior polarity. They appear to be more closely related to the true bacteria than to the protozoa and may, perhaps, be regarded as a connecting link between the two.

Classification and nomenclature within the group are still in an uncertain condition, and there is by no means general agreement regarding the separation of some of the genera. These microorganisms are difficult to cultivate on artificial media in many cases, and little or nothing is known of their physiological characteristics; the genera are separated from one another on a purely morphological basis.[1]

Although the name spirochete is commonly used to designate those forms which are classified as members of the family Spirochaetaceae, the generic term Spirochaeta was originally proposed by Ehrenberg[2] for the large free-living forms he described. A second genus, Cristispira, is characterized by the presence of a membranous appendage or crista wound about the body of the cell, and is made up of microorganisms found living as saprophytic commensals in certain molluscs. A third genus, Saprospira, consists of free-living forms which differ from Cristispira in that the crista is absent. The other genera contain the spirochetes which are pathogenic for man and animals; Borrelia, the spirochetes of the relapsing fevers; Leptospira, the spirochetes causing Weil's disease or infectious jaundice and certain fevers; and Treponema, the spirochetes of syphilis and yaws. The differential characteristics of these genera are summarized in the accompanying table.

[1] *Cf*. Davis: Ann. Rev. Microbiol., 1948, 2:305.
[2] Ehrenberg: *Die Infusionstierchen*. 1838.

THE SPIROCHETES OF THE RELAPSING FEVERS (BORRELIA)[3]

The relapsing fevers are a group of closely related infections characterized clinically by an initial pyrexia of three to four days' duration, followed at intervals of a few days by successive relapses. They are widely distributed and occur in every country of the world with the possible exception of Australia. The microorganisms responsible for these diseases are spiral forms, first observed by Obermeier[4] in the blood of patients with European relapsing fever.

Morphology. The basic structure of these spirochetes is a springlike axial filament upon which there is a layer of contractile protoplasm enveloped in a delicate periplast. The terminal filaments are, perhaps, drawn-out ends of the periplast or non-rigid and non-coiled ends of the axial filament. Attached
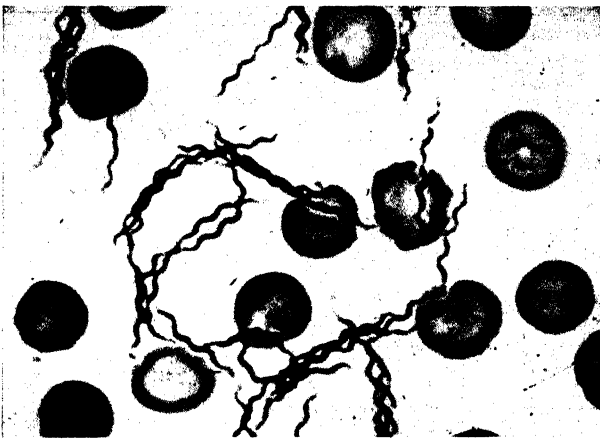
Fig. 195. *Borrelia recurrentis* in blood smear. × 2000 (Kral).

spherical bodies are sometimes observed and these are thought to be minute extrusions of cytoplasm through the periplast. The cell rotates as a consequence of the stretching of the axial filament by the pressure of contracting protoplasm. When there are rapid and successive contractions and relaxations, the microorganism rotates rapidly and moves in one direction or another according to the direction of the rotation. In the relaxed state the spirals are regular and even; the larger the cell the heavier the axial filament and the greater the distance between the spirals. The regularity of the spirals is disturbed by the contracting and relaxing protoplasm in the actively motile cells. The various species cannot be distinguished from one another on a morphological basis. All are 0.2 to 0.5 μ in breath by 10 to 20 μ in length. They are best stained by the Romanowsky method or some modification such as Giemsa, but, in contrast to some of the others, Borrelia may be stained with the ordinary aniline dyes.

Cultivation. The spirochetes of relapsing fever have been cultivated by Noguchi[5] in a medium of ascitic fluid and fresh tissue in which a network

[3] For general discussions see A.A.A.S. Symposium No. 18, *Relapsing Fever in the Americas*, 1942; Hemingway, Hemingway and Arneson: Northwest Med., 1940, 39:362.
[4] Obermeier: Centralbl. f. d. med. Wissenschr., 1873, 11:145.
[5] Noguchi: Jour. Exp. Med., 1912, 16:199, 620.

CLASSIFICATION OF SPIRAL MICROORGANISMS

	Spirochaeta ("Coil-hair")	Saprospira ("Putrid-coil")	Cristispira ("Crested coil")	Borrelia (Borrel: Compt. rend. Soc. biol., 1906, 60:138)	Treponema ("To Turn, Thread")	Leptospira ("Fine-coil")
Measurements in microns — Length	100 to 150	100 to 120	45 to 90	8 to 16	6 to 14	7 to 9 or 14
Diameter	0.5 to 0.7	....	1.0 to 1.5	0.35 to 0.5	0.25 to 0.3 Cylindrical	0.25 to 0.3
Width of spiral	2	....	....	....	1.0 Regular, rigid	0.45 to 0.5
Length of spiral	1.5 Regular	....	....	....	0.8 to 1.0 Very constant	0.3 Regular
Shape of ends	Blunt	....	Blunt	Pointed	Pointed	Pointed
Waves (in addition to finer spirals).	Several large, inconstant, irregular.	Large, inconstant, shallow, irregular, 3 to 5 in number.	Two to five or more. Large, irregular, shallow.	Large, wavy spirals, usually five.	One or more slight undulating waves.	One or more gentle wavy curves throughout entire length. One or both ends semicircularly hooked when in liquid media. Serpentine in semisolid media. Extremely flexible.
Axial filaments	Present (flexible elastic)	Absent	Absent	Present	Present	Absent
Chambered structure	Absent	Present	Present	Absent	Absent	Absent

Membrane..............	Absent	Present (flexible elastic)	Present (flexible elastic)	Delicate, flexible, double contoured.	Doubtful	Absent
Terminal (finely spiral filament).....	Absent	Absent	Absent	Present	Present	Not recognized
End (highly motile end portion).	Absent	Absent	Absent	Absent	Absent	Well developed in last 6–8 spirals.
Crista (undulating membrane).	Absent	Absent	Present; ridge-like membrane spirally about body.	Absent	Absent	Absent
Division (character)........	Transverse	Transverse	Transverse	Transverse, possibly also longitudinal.	Transverse, possibly also longitudinal.	Transverse
Habitat............	Fresh or marine water.	Foraminiferous sand.	Parasitic in alimentary canals of shell fish.	Numerous pathogenic and non-pathogenic varieties.	Three pathogenic and several harmless parasites.	Two pathogenic varieties and one possibly non-pathogenic.
Action of chemicals: Trypsin digestion............	Axial filament resistant.	....	Membrane resistant. Crista and chambers disappear.	....	Resists for many days.	....
Bile salts (10 per cent).....	Becomes shadowy, pale, but is not dissolved.	....	Crista destroyed. Body not attacked.	Complete disintegration.	Complete disintegration.	Easily dissolved.
Saponin (10 per cent).....	Lives thirty minutes; later becomes shadowy, pale, but not dissolved.	....	Crista becomes fibrillar and then indistinct. Body not affected.	Immobilized in thirty minutes. Broken up in a few hours.	Eventually broken up.	Completely resistant.

of fibrin is formed. A semisolid serum agar has been used by Kligler and Robertson.[6] In culture the spirochetes are aerobic and the paraffin-oil seal frequently used prevents evaporation rather than interferes with the diffusion of oxygen. While such successful cultivation has been reported, the spirochetes are exceedingly difficult to grow in initial culture and cannot be maintained in serial passage; it is not unlikely that they only persist for a time in such media and never have been actually cultivated. They may be grown in the developing chick embryo,[7] however, and have been isolated directly from human infections in this way.[8]

Classification. The relapsing fever spirochetes have been given the generic name Borrelia and are so called in the Bergey classification. The validity of this degree of differentiation from the spirochetes of syphilis and yaws,
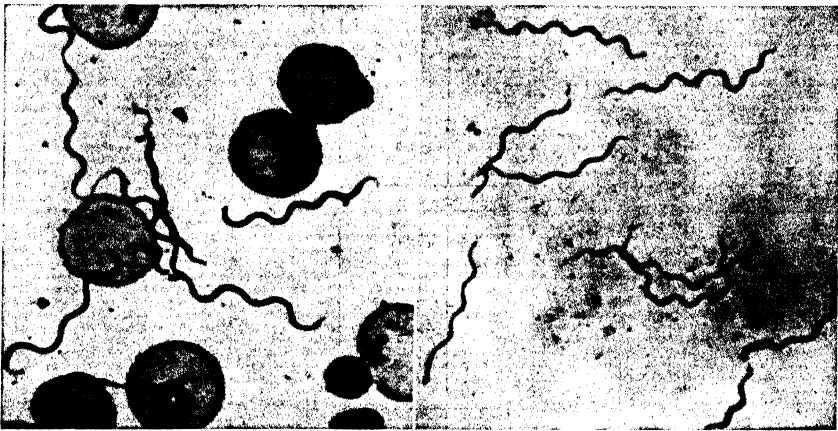
Fig. 196. Relapsing fever spirochetes. Left, *Borrelia duttonii* of Central African relapsing fever; right, *Borrelia kochii* of East African relapsing fever. × 2000 (Kral).

Treponema, is open to some question, and the relapsing fever spirochetes are regarded by many as Treponema species. It has been pointed out by Noguchi that the relapsing fever spirochetes do not differ more from *Treponema pertenue* (yaws) than that microorganism differs from *Treponema pallidum*.

It is not clear to what extent the relapsing fever spirochetes can be divided into species. It is probable that in many cases those given species rank are, in fact, only strains or varieties. In many instances these have been given place names or named after some individual worker but the differentiation has been geographical rather than biological. These forms are immunologically heterogeneous and unstable in that they are modified in character by residence in different insect or mammalian hosts. Cross-immunity tests, therefore, are not useful. It has been suggested that they are differentiated on the basis of pathogenicity or insect host but there appear to be no reasonably definite lines of demarcation possible on these bases. In general, then, the questions of interrelationship and speciation of these organisms are almost completely open

[6] Kligler and Robertson: Jour. Exp. Med., 1922, 35:303.
[7] Chabaud: Bull. Soc. Path. Exot., 1939, 32:483; Oag: Jour. Path. Bact., 51:127.
[8] Bohls, Irons and DeShazo: Proc. Soc. Exp. Biol. Med., 1940, 45:375.

at present, but most workers agree that, although they are all immunologically related, there is too great heterogeneity to justify regarding the various strains as no more than varieties of a single species *Borrelia (Treponema) recurrentis.*

Pathogenicity. As indicated above, the relapsing fevers constitute a group of closely related diseases. The spirochete observed by Obermeier was that of European relapsing fever, a disease which has been known since the early part of the eighteenth century and has at times prevailed extensively in parts of Europe. The causal microorganism is designated *Borrelia* (or *Treponema*) *recurrentis.* Four forms of relapsing fever occur in Africa; the spirochete of the form in Central Africa is *Borrelia duttonii,* that of the East African disease *Borrelia kochii,* that occurring in northern Africa, in Tunis, Algiers and Tripoli is *Borrelia berbera,* and that in the Sudan *Borrelia aegyptica.* Relapsing fever also occurs in India, and the causative microorganism is *Borrelia carteri.* American relapsing fever is caused by still another spirochete, *Borrelia novyi;* other strains isolated in this country have been named *Borrelia turicatae.* South American strains have been called *Borrelia venezuelensis* and *Borrelia neotropicalis.*

All forms of relapsing fever are clinically identical. The onset is sudden, with chills, fever and severe headache, muscular and joint pains are frequently observed, and there is a moderate enlargement and tenderness of the spleen. The fever ends suddenly by crisis in three or four days. Successive relapses occur at intervals of two to fourteen days, and the period of relapse varies from a few hours to longer than the primary fever. The number of relapses varies. Spirochetes may be found in the blood during the paroxysms. The case fatality is not high in European relapsing fever, perhaps 4 to 5 per cent. There are no characteristic findings at autopsy.

The infection may be transmitted to the rat and mouse, but the guinea pig appears resistant to most strains though it may be infected with certain American strains.[9] It has been reported that the hamster may be infected with at least some strains.[10] It is of some importance that the blood for inoculation be taken at the onset rather than at the decline of the relapse. Ordinarily the infection is inapparent in both rats and mice in that there are no observable symptoms, but successive "attacks," beginning two to four days after inoculation, are evidenced by the appearance of the spirochetes in the blood. They are present for two to three days, disappear, and reappear three or four days later in a second "attack." Usually only two or three such relapses occur, the spirochetes becoming fewer and persisting for shorter times in the successive relapses. Experimental disease may also be produced in monkeys and its course is very similar to that in man.[11]

Natural infection of lower animals occurs with some frequency in endemic areas and there is much evidence to support the hypothesis of Nicolle and Anderson[12] that small mammals, especially rodents, constitute a natural reservoir of the tick-borne infection. A wide variety of animals may be infected,

[9] Davis: Pub. Health Repts., 1939, 54:1721.

[10] Chen and Anderson: Proc. Soc. Exp. Biol. Med., 1941, 46:658.

[11] Experimental infections have been studied in detail by Novy and Knapp: Jour. Inf. Dis., 1906, 3:291.

[12] Nicolle and Anderson: Bull. Inst. Pasteur, 1927, 25:657.

including the rat, mouse, chipmunk, squirrel, opossum, porcupine, armadillo, hedgehog, fox and dog. Susceptibility varies considerably among animal species, and from one strain to another of the spirochete.

A spirochetosis of geese is due to *Borrelia anserinum*, a microorganism regarded by many as identical with *Borrelia gallinarum*, which produces a septicemia of chickens. Another species, *Borrelia theileri*, is associated with a benign affection of cattle in South Africa but appears to have but low pathogenic powers. Morphologically similar spirochetes have also been found in the blood of sheep, horses and bats and in the alimentary tract of fish and insects, but they are not associated with any obvious indisposition.

Immunity. Following infection agglutinins, lytic and spirocheticidal antibodies may be found in the serum. Usually the immunity following recovery from an attack of relapsing fever is of short duration but occasionally a more solid immunity may develop. The latter is often attributed to a persistence of the infection, *i.e.*, an immunity to superinfection.[13] This is not definitely known, however.

Relapse appears to be basically an immunological phenomenon and dependent upon the antigenic instability of the spirochete. It was shown by Novy and Knapp[11] that the spirochetes will remain alive for as long as forty days in blood drawn before the onset of an attack, but in blood drawn during the decline of an attack or after recovery they die out in less than an hour. In the latter case the blood is spirocheticidal and the killed spirochetes are phagocytosed. It was suggested by Novy[14] that the relapse is a consequence of the survival of a few individuals which are resistant to the specific spirocheticidin and which multiply to give rise to a new serum-fast strain. It has been found by Cunningham and his co-workers[15] and by Meleney[16] that spirochetes isolated after successive relapses differ serologically from the spirochetes of the first attack, and as many as nine different serological types of *Borrelia carteri* were distinguished by Cunningham; after any particular type appeared in an animal, it never reappeared in the further course of the disease in the same animal. It is not clear whether this immunological variation is "normal" in the sense that it occurs without environmental stimulus as in the case of the phase variation of Salmonella (p. 439), the antiserum selecting out the resistant immunological type, or whether it is the kind of induced variation produced by cultivation of a microorganism in the presence of homologous antibody to give rough variants.

The parallel between this and the successive relapses of certain types of trypanosomiasis is striking. In the guinea pig infected with *Trypanosoma rhodesiense* recovery is due to the development of a specific trypanolysin, the relapse to the multiplication of serum-fast trypanosomes, second recovery to a second specific trypanolysin and so on. In this case the serum-fast strains are immunologically distinct from one another. In *Trypanosoma lewisi* infections in the rat, however, both a trypanolysin and a reproduction-inhibiting anti-

[13] *Cf.* Hindle: Trop. Dis. Bull., 1935, 32:309; Coleman: Jour. Inf. Dis., 1934, 54:1; Beck: Jour. Inf. Dis., 1937, 60:64.

[14] Novy: Science, 1908, 27:650.

[15] Cunningham: Trans. Soc. Trop. Med. Hyg., 1925, 19:11; Cunningham *et al.*: Indian Jour. Med. Res., 1934, 22:105, 595.

[16] Meleney: Jour. Exp. Med., 1928, 48:65.

body, or ablastin, are formed; the inhibition of cell division does not, presumably, allow the development of serum-fast strains, and hence there are no relapses.[17]

Diagnosis.[18] The diagnosis of relapsing fever is made by demonstration of the spirochetes in the blood during the onset of a relapse by direct microscopic examination or by animal inoculation. Either the usual blood smear or a thick film similar to that used for the detection of malarial parasites may be used. The films are air-dried and stained with Giemsa; Wright's stain is satisfactory for thin films. The spirochetes may also be found in fresh wet preparations by darkfield examination. Mice and/or rats may be inoculated intraperitoneally and blood smears made daily for as long as two weeks if the spirochetes are not found.

In the past immunological methods of diagnosis have not been satisfactory. More recently, however, Stein[19] has prepared spirochete antigens by saponin hemolysis and washing of the spirochetes. Complement fixation, said to be preferable for diagnostic purposes, and agglutination with sera from infected persons or animals occur and are reported to be specific.

Epidemiology. The relapsing fever spirochetes are blood rather than tissue parasites and the infection is transmitted by blood-sucking insects, though rare cases of direct transmission may possibly occur. Two epidemiological types of the disease are to be distinguished, the one tick-borne and representing transmission from an animal reservoir of infection to man, and the other louse-borne infection spread from man to man.

A tick vector was not recognized in this country until 1930[20] but the tick-borne disease is now known to be not uncommon and at the present time is the only type which occurs. Known endemic foci of infection exist in Arizona, California, Colorado, Idaho, Kansas, New Mexico, Nevada, Oklahoma, Oregon and Texas and possibly also in Montana, Utah and Washington. *Ornithodorus turicata* and *O. hermsi* are known to transmit the disease and it seems probable that *O. parkeri* transmits it also but final proof is lacking. *O. talaje* transmits the infection in tropical America but apparently not in the United States; *O. rudis* is regarded as the most important vector in the tropics in this hemisphere. The traditional vector, and the most common in West Africa, is *O. moubata*. There appears to be no specific relationship between any particular species of tick and any particular strain of spirochete.

Infection may persist for long periods of time in the tick; Francis[21] has reported survival in starved ticks for as long as five years, and in refed ticks for six and one-half years. Furthermore, the spirochete may be transmitted to the offspring and even the third generation may be infected. The spirochetes are present in the coxal fluid, saliva and feces of infected ticks. Of these, the first two appear to be the more important in transmission of the infection to man. For example, *O. moubata*, an excellent vector, secretes coxal fluid copiously while feeding, thus making possible infection of the bite, while *O. hermsi*,

[17] *Cf.* Taliaferro: Jour. Exp. Med., 1924, 39:171.
[18] *Cf.* Bohls and Schuhardt: Texas State Jour. Med., 1933, 29:199.
[19] Stein: Jour. Exp. Med., 1944, 79:115.
[20] Weller and Graham: Jour. Amer. Med. Assn., 1930, 95:1834.
[21] Francis: Pub. Health Repts., 1938, 53:2220.

which is not known to transmit the infection to man though it does so from rat to rat, does not pass coxal fluid while feeding. The relative importance of direct introduction of the spirochete into the bite by infected saliva is not altogether clear. It is not improbable that infection of the bite by contaminated feces occurs occasionally.

European relapsing fever is transmitted from man to man by the human body louse, *Pediculus vestimenti,* and has the epidemiological characteristics of louse-borne disease. In contrast to the tick-borne infection, the bite is not infected by the secretions of the lice; rather the infected louse must be crushed on the skin and the spirochetes present in the body fluids contaminate the
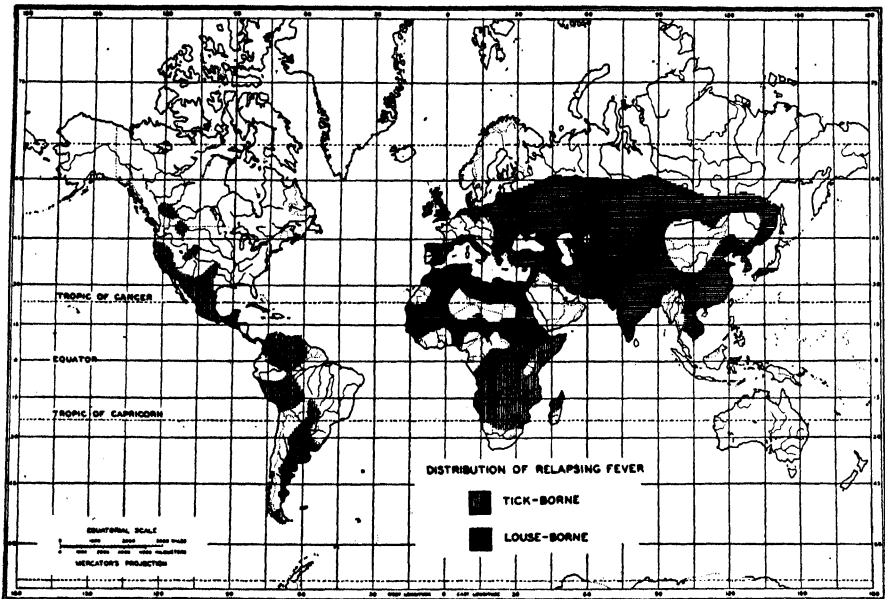
Fig. 197. The world-wide distribution of relapsing fever, both louse- and tick-borne. Redrawn from map prepared by Army Medical Intelligence, 1943. Based on Goode *Base Map* No. 201M. By permission of the University of Chicago Press.

bite. The infection is transmitted to the egg, and there is some evidence that the disease may be produced by crushed eggs laid by infected lice.

It is possible that the fingers may become contaminated by crushing infected lice and transmit the infection by scratching. The bedbug may also serve as a mechanical carrier of the infection.

Spirochetosis of fowls is transmitted by a tick, *Argas persicus,* which infests fowls in the warmer parts of the world and, as in human relapsing fever, the spirochete is transmitted through the egg of the tick to the offspring.

VINCENT'S ANGINA (BORRELIA VINCENTII)[22]

Vincent's angina, an ulceromembranous angina and stomatitis of the mucous membranes of the mouth and throat, is variously known as Plaut-

[22] See the review by Weaver: Med. Res. Council (Great Britain) Monthly Bull., 1943, 2:107.

Vincent's angina, pseudomembranous angina or trench mouth. The symptoms and lesions are quite characteristic, and the affection is prevalent, though it generally passes unnoticed because of its mild character.

Smears from the necrotic lesions show two types of bacteria intermingled, fusiform bacilli (p. 542) and spirochetes. The latter are morphologically similar to the relapsing fever spirochetes and have been designated *Borrelia vincentii*. The relation of the spirochetes to the fusiform bacilli is by no means clear. It has been supposed by some that the two are distinct microorganisms, closely associated in some sort of symbiotic relation. Others[23] are of the opinion that these two forms are different phases in the life history of a single microorganism. The balance of the evidence is, however, against the latter view. The fusiform bacilli may be cultivated as indicated in an earlier chapter (Chap. 26), but as yet the spirochetes have not been grown on artificial media.
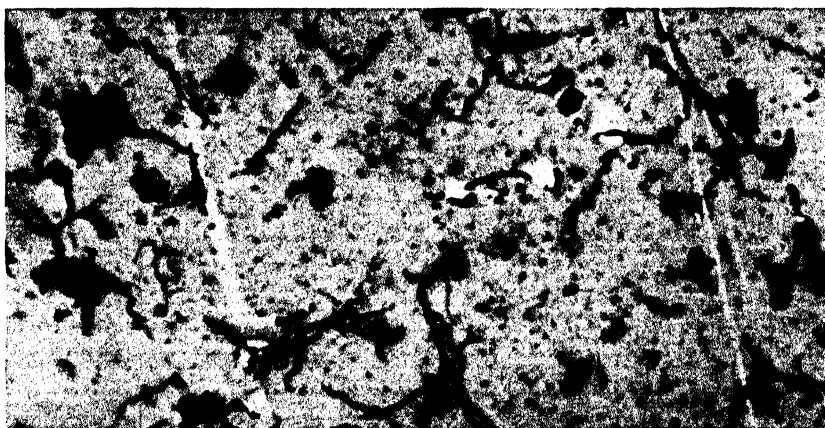
Fig. 198. Smear from Vincent's angina showing the characteristic mixture of spirochetes (*Borrelia vincentii*) and fusiform bacilli. Fuchsin; × 1250 (P. E. Harrison).

The causal relation of these microorganisms to Vincent's angina is not established. They are found in practically every normal mouth and in a variety of pathological conditions where their presence suggests that of a secondary invader rather than that of a primary exciting cause. Pure cultures of the bacilli injected into traumatized tissues have failed to produce lesions.[24] It has been found that dogs injected with a squill glucoside (scillaren B) develop a condition of the mouth identical with that of Vincent's angina. The etiology of this disease is as yet entirely unknown, as is the pathogenicity of the spirochetes of the mouth.[25]

SYPHILIS (TREPONEMA PALLIDUM)

Syphilis was not recognized as a clinical entity prior to 1493. The origin of the disease has been a matter of dispute, some maintaining that it was taken

[23] Tunnicliff: Jour. Inf. Dis., 1906, 3:481.

[24] Lichtenberg, Werner and Lueck: Jour. Amer. Med. Assn., 1933, 100:707.

[25] For discussions see Hanell: Arch. Otolaryng., 1931, 14:1; Smith: *Oral Spirochetes and Related Organisms in Fuso-Spirochetal Diseases.* Williams & Wilkins Company, Baltimore. 1932; Gins: Ztschr. f. Hyg. u. Infektionskr., 1942, 124:460.

to Europe from the New World by the crew of Columbus, and others that it was already present in Europe.[26] In any case, some of the returning crew accompanied Charles VIII of France in the invasion of Italy in 1494. The epidemic which began in Italy at this time spread over Europe with the scattering of the troops. The sexual transmission of the disease was not realized until some time after this epidemic outbreak. The French referred to it as the Italian disease, and the Italians termed it the French disease or *morbus gallicus.* The disease derived its name from the principal character of the poem "*Syphilis sive Morbus Gallicus*" written by Fracastorius in 1530.[27]

The disease was transmitted to monkeys and anthropoid apes by Metchnikoff and Roux[28] in 1903, and the spirochete was described by Schaudinn and Hoffmann[29] in 1905.

Morphology. As indicated earlier, the morphological differences between Borrelia and Treponema are minor, and *Treponema pallidum* need not be further described here. Involution forms and granular forms may be seen[30] and Levaditi[31] believes that granules are viable and infectious. This spirochete is exceedingly difficult to see in the unstained state by transmitted light and is also refractory to stains. It may be observed unstained in fresh preparations in the darkfield and in smears by transmitted light in india-ink relief stains. The spirochete may be stained in smears by Giemsa's eosin azure and in tissue sections by the silver impregnation methods used in neurological work. Somewhat simpler methods of staining with Victoria blue or carbol-gentian violet are reported to be satisfactory.[32]

Cultivation. Schereschewsky[33] reported the cultivation of *T. pallidum* on artificial media in 1909, but his cultures appear to have been contaminated with other microorganisms. Noguchi[34] later reported the growth of several

[26] See Holcolm: Bull. Hist. Med., 1941, *10*:148.

[27] "The other poem, also by Fracastor, entitled *Siphilis*, dedicated to the future cardinal, Pietro Bembo—the same who shunned reading the Epistles of Paul the Apostle lest he corrupt his own style—hymned forth in line just as irreproachably elegant as the first, and also after Virgil, the French Malaise and the modes of treatment by sulphur baths and mercury ointment. The origin of the disease was explained, *in passim,* by the fable of a certain shepherd of ancient times by the name of Siphilis, who had aroused the ire of the god of the Sun with his mockeries; the god did punish him with a malady which would not yield to any treatment, until the nymph America did initiate him into her mysteries and did lead him to a grove of the healing Guaiacum trees, a sulphur spring, and a lake of mercury. Subsequently Spanish travelers, having sailed across the ocean and discovered the New Lands, wherein the nymph America dwelt, did also offend the god of the Sun, having shot in the chase the birds sacred to him, of which one did prophesy in a human voice that for this sacrilege Apollo would send them the French Malaise." Merejkowski: *The Romance of Leonardo da Vinci.* Guerney translation, Modern Library.

[28] Metchnikoff and Roux: Ann. Inst. Pasteur, 1903, *17*:809; *ibid.,* 1904, *18*:1, 657; *ibid.,* 1905, *19*:673.

[29] Schaudinn and Hoffmann: Arb. a. d. k. Gesund., 1905, 22:527; Deut. med. Wchnschr., 1905, *31*:711.

[30] See the detailed morphological studies of Manouelian: Ann. Inst. Pasteur, 1940, 64:439.

[31] Levaditi: C. R. Soc. Biol., 1941, *135*:467; see also Hampp, Scott and Wyckoff: Jour. Bact., 1948, 56:755.

[32] Goldsworthy and Ward: Jour. Path. Bact., 1942, 54:382.

[33] Schereschewsky: Deut. med. Wchnschr., 1909, 35:835.

[34] Noguchi: Jour. Exp. Med., 1911, *14*:99.

strains of the spirochete in pure culture under strictly anaerobic conditions in a serum water medium containing a piece of sterile fresh rabbit kidney or testicle. These cultures were carried through a number of generations and produced typical lesions on intratesticular inoculation in the rabbit. The spirochete is very difficult to grow, especially in the first generation from the animal body. Noguchi first grew it from syphilitic lesions in the rabbit but was later able to cultivate it directly from man. Culture in the developing chick embryo is not very successful.[35] Culture of the spirochete is, therefore, not a feasible routine procedure, and the microorganism is generally kept in the laboratory in infected animals. The spirochetes remain alive indefinitely in the lymphatics of the rabbit and may be readily obtained by excision of a popliteal gland. It has been found[36] that the microorganism also remains viable indefinitely in the mouse and may be recovered from the lymphatics, spleen or
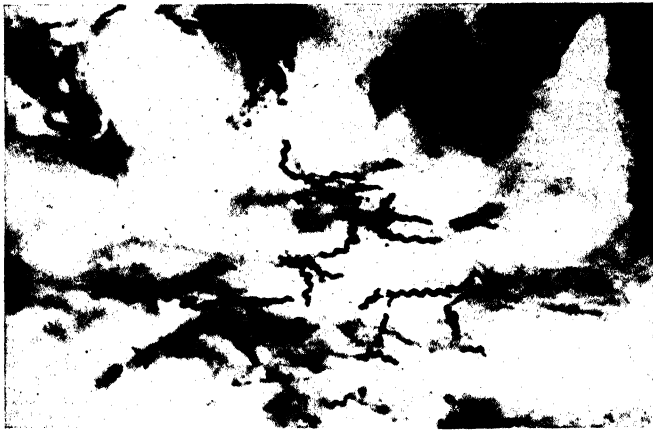
Fig. 199. *Treponema pallidum* in stained (silver impregnation) smear. × 2000 (Kral).

brain. Levaditi[37] is of the opinion that different strains differ in their pathogenicity for experimental animals.

T. *pallidum* is a very fragile microorganism and dies out quickly outside the body. It is readily killed by drying and is unusually susceptible to heat; suspensions of infected rabbit testicle are sterilized by exposure to 41.5° C. for one hour and 41° C. for two hours. In undisturbed cultures kept uninterruptedly at 37° C. it may remain alive for as long as a year. Like other bacteria, this spirochete is much less affected by extreme cold. It remains viable for at least a year in infected tissue frozen at −78° C. and stored at that temperature; stored at −20° C., however, it dies out in forty-eight hours.[38] The risk of transmission of syphilis by blood from blood banks is negligible for the spirochete dies out in three days at refrigerator temperature,[39] and

[35] Callaway and Sharp: Jour. Lab. Clin. Med., 1941, 27:232; Wile and Snow: Jour. Invest. Dermat., 1941, 4:103; *ibid.*, Amer. Jour. Syph., 1944, 28:187.

[36] Kolle and Schloszberger: Deut. med. Wchnschr., 1928, 54:129.

[37] Levaditi: Ann. Inst. Pasteur, 1942, 68:118.

[38] Ravitch and Chambers: Bull. Johns Hopkins Hosp., 1942, 1:299.

[39] See the discussion in the Lancet, 1941, i:286.

rapidly in lyophilized plasma[40] but may persist for a longer time in frozen blood.

Pathogenicity for Man. Syphilis is transmitted from person to person through intimate contact, usually sexual. Indirect transmission may occur, however, through the agency of articles such as common drinking cups, towels and the like, if the time interval is very short. The spirochete penetrates the skin and mucous membranes through minute abrasions and possibly through the intact skin, though this is not definitely known. An appreciable lapse of time is required for penetration, however, for it was early shown by Metchnikoff and Roux[28] that the application of calomel ointment one to two hours after cutaneous inoculation of chimpanzees prevented infection.

The prevalence of syphilis is not known definitely; the estimate that 10 per cent of adults in the United States will give positive Wassermann tests is probably fairly accurate. It is also estimated that between 1 and 2 per cent of the children in this country have congenital syphilis. The mortality from syphilis is also not known. The death rate of 13 to 14 per 100,000 is misleading, for deaths reported as due to other causes are frequently due to syphilis; all cases of general paresis, locomotor ataxia and many cases of apoplexy and aortic aneurysm are, for example, due to syphilitic infection, and the disease is a contributory cause in deaths from other diseases. Studies on the incidence of syphilis in autopsies on adults have given rates from 2.6 per cent to 29.5 per cent; the average in 146,761 autopsies performed from 1896 to 1938 was 5.45 per cent.[41]

In this country the prevalence is much higher in the Negro than in the white; tests of the first two million selectees under the Selective Service Act showed 2 per cent in white and 22 per cent in Negroes. There is considerable variation in the ratio from one part of the country to another.[42] Smillie,[43] therefore, regards syphilis in the United States as primarily a Negro problem.

A disease of protean manifestations, syphilis in man assumes several relatively well-defined clinical stages. The ordinary course of the disease includes the successive appearance of the so-called primary, secondary and tertiary stages; congenital syphilis is best regarded as a separate subdivision.

Primary Stage. Following the penetration of the skin or mucous membranes, the spirochetes rapidly invade the tissues; it has been found that the inguinal lymphatics of rabbits become infective thirty minutes after cutaneous inoculation of the scrotum.[44] The initial primary lesion appears ten to thirty days following infection. The typical hunterian chancre (so called because it was described accurately and in detail by John Hunter) is an indolent, indurated ulcer, single and painless, and not infrequently atypical and trifling in appearance. The presence of the spirochetes in the chancre serum may be demonstrated by darkfield examination and constitutes the diagnostic method in early primary syphilis, for a positive Wassermann reaction (see below) does not develop until two or three weeks after infection. Clinically, then, syphilis

[40] Probey: Pub. Health Repts., 1947, 62:1199.

[41] These studies have been summarized by Rosahn and Black-Schaffer: Arch. Int. Med., 1943, 72:78.

[42] See Greve: Amer. Jour. Public Health, 1946, 36:751.

[43] Smillie: Jour. Amer. Med. Assn., 1943, 122:365.

[44] Kolle and Evers: Deut. med. Wchnschr., 1926, 52:1075.

in the primary stage is a localized infection, but bacteriologically it is a generalized infection, and spirochetes are often present in the blood stream.

Secondary Stage. In the secondary stage the generalized nature of the infection is apparent. Bones, joints, eyes and other organs are invaded with the appearance of constitutional symptoms, cutaneous lesions, enlargement of the lymph glands, pains in the joints and the like. Evidences of the development of this secondary stage are usually apparent four to eight weeks after the appearance of the chancre but may be delayed for a year or more. Spirochetes are present in all the lesions and may be found in the blood stream.

Tertiary Stage. The disease passes insensibly into the tertiary stage and may persist for years. Ulcerating necrotic lesions of the skin and gummata of the internal organs are common, and the clinical manifestations of infection are so highly diverse that there is no characteristic picture. Spirochetes are present in the tertiary lesions but only in small numbers.
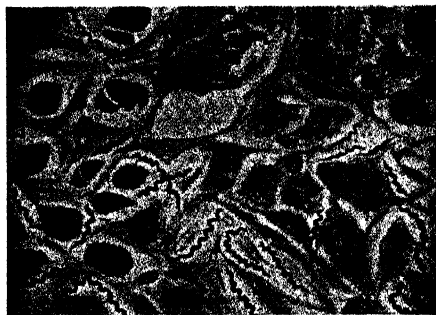
Fig. 200. *Treponema pallidum* in congenital syphilis; × 1000; Levaditi (Sobernheim, in Kolle and Wassermann).

Neurosyphilis. Spirochetal invasion of the central nervous system may occur independently of the so-called stages and as soon as the early secondary stage. Generally, however, neurosyphilis is a manifestation of late tertiary infection and may appear years after the initial infection, and tabes dorsalis, general paresis and other evidences of the invasion are sometimes termed quaternary or parasyphilitic affections. The spirochetes may be found in the cord in tabes dorsalis and in syphilitic brains in the cortex, in the pia and vessel sheaths. General paresis is, then, a diffuse spirochetosis of the brain involving the cortical layers chiefly, and the presence of the spirochetes in the parenchyma explains much of the histopathological picture and the nervous symptoms of the affection.

Latent Syphilis. In some instances the primary lesion is not noticed and the secondary manifestations may be slight, and with their subsidence there is no clinical evidence of disease and the infection remains latent within the tissues. In other instances the latent condition develops after primary and secondary symptoms of usual severity. Such clinically latent infections may, perhaps, be regarded as the maintenance of a biological balance between the host and the parasite. Latent infections may, of course, develop clinical manifestations at a later time.

Congenital Syphilis. The fetus is readily infected *in utero* by spirochetes

passing through the placenta. The infection may result in a cessation of development and abortion, or the fetus may go to term and be born dead. In those who survive, the disease is generalized from the beginning, and the lesions are those of tertiary syphilis. Spirochetes may be demonstrated in great numbers in the tissues and organs affected. Third generation syphilis has been reported but is very rare.[45]

Immunity. At the present time syphilis is not so severe a disease in man as it was in the early sixteenth century. Whether this is an expression of an adaptive response on the part of man with the development of a low degree of natural immunity or a similar response on the part of the parasite manifested as a decrease in virulence, is not known; possibly both may have occurred.

The specific immune response to syphilitic infection is not well understood. There is a marked apparent insusceptibility to reinfection that is illustrated by the fact that a second chancre may be produced by reinfection prior to the appearance of the first chancre, but after the first chancre has appeared further reinfection does not produce an initial lesion. It is commonly stated that man, once infected, is refractory to reinfection and that reinfection occurs only very rarely. In other words, there is an infection-immunity or immunity to superinfection superficially similar to that observed in tuberculosis. The refractory state may persist after "cure," but there is no assurance that the disease may be "cured" in the sense that the spirochete is completely eliminated from the tissues.

Evidence obtained in animal experiments suggests that this immunity may be more apparent than real. It has been shown, for example, that spirochetes inoculated after the development of primary lesions gain access to the tissues even though they produce no new primary lesion. Whether a similar situation holds in man and the observed refractory state is only a "clinical immunity" or whether there is a genuine immune response is not clear.

Serodiagnosis.[46] A type of antibody activity, attributed to the presence of a substance designated *reagin*, is developed as a consequence of infection with *T. pallidum* which has great practical value since it allows the serological diagnosis of syphilis. A complement-fixation test was proposed by Wassermann and his co-workers in 1906 as an immunologically specific reaction, using a watery extract of syphilitic fetus as the antigen. It was soon found, however, that syphilitic sera fix complement in the presence of an extract of normal tissue.

Not long after the development of the Wassermann test it was observed that syphilitic sera produce a flocculation when mixed with Wassermann antigen. The reacting substance present in the antigen preparations occurs in various tissues but heart (beef) muscle extracts are the most satisfactory. It is soluble in alcohol and insoluble in acetone, and its activity is considerably increased by the addition of cholesterol. Activity of these extracts is due to a phospholipid, *cardiolipin*, which is precipitated from the initial alcoholic extract with cadmium chloride, freed from lecithin and fractionated by precipitation from ether with acetone and alcohol. Anticomplementary activity of the preparation

[45] See summary of reported instances by Maijala: Acta Med. Scand., 1940, 103:1.

[46] For a detailed and critical discussion see *Modern Serologic Tests for Syphilis*. Ven. Dis. Inform. Suppl. No. 14, 1941; also the critical review of recent literature by Osmund: Bull. Hyg., 1946, 21:627.

is removed by the addition of lecithin, and complement is not fixed unless cholesterol is added. According to Pangborn,[47] who described this substance in 1941, it is a complex phosphatidic acid.

Types of Tests. Probably no serological reactions have been as intensively studied as the complement-fixation and precipitation tests for syphilis, and the techniques have been modified and refined by many workers to increase sensitivity and specificity. At the present time two complement-fixation tests, the Kolmer test and the Eagle complement-fixation test, and four precipitation tests, the Kahn test, the Kline test, the Hinton test and the Eagle precipitation test, are used.[48] Of these the most common are the Kolmer complement-fixation and the Kahn precipitation tests. Detailed studies have shown that all these tests agree quite well, disagreement being most common with treated cases. In addition to these, more sensitive precipitation tests have been devised by Kahn and by Kline and are known as the Kahn presumptive test and the Kline exclusion test; they are apt to give false positive reactions but are regarded by some workers as having considerable utility as screening tests. Furthermore, the complement-fixation test may also be quantitative, *i.e.*, the reagin present in the syphilitic serum may be titrated. For ordinary diagnosis this is unimportant since it is not known whether the reagin titer has any significance, but it is useful in following therapy and in the diagnosis of congenital syphilis in infants. In the latter case reagin is passively transferred from the maternal circulation; if the infant is not infected the reagin titer drops slowly, but a gradually increasing titer is indicative of infection. The usual qualitative complement-fixation or precipitation test is used in the so-called provocative test in which a suspected latent syphilitic infection in a serologically negative individual is activated by a small dose of an arsenical, the test being run at intervals thereafter for ten days or more. All these tests may be, and frequently are, done with spinal fluid in cases of infection of the central nervous system.

The Effect of Therapy. The majority of individuals undergoing therapy become serologically negative in time and this response is usually taken as an indication of the efficacy of the treatment. The time required to attain negativity is variable and depends upon the individual, the stage of the disease, the course of therapy, especially if continuous or intermittent, and the sensitivity of the serologic test used. No general statement of any precision is possible, therefore, but it is usually said that the majority of persons with primary or early secondary syphilis become negative after one or two courses of arsenical or continuous treatment for five to seven weeks. Some, however, become negative with only one or two injections of drug, while others may remain positive for months and a certain proportion remain positive indefinitely. The last is spoken of as Wassermann-fast, reagin-fast, or seroresistant syphilis. Slightly over 10 per cent of infections are in this group. Two explanations are usually offered for seroresistant syphilis, one that persistent foci of infection remain, and the other that the continued presence of reagin is evidence of a definite immune response.

[47] Pangborn: Proc. Soc. Exp. Biol. Med., 1941, 48:484; Jour. Biol. Chem., 1947, 168:351.

[48] Detailed instructions for the performance of these tests may be found in Ven. Dis. Inform., Suppl. No. 11, 1940.

Specificity and False Reactions. It will be apparent that the serological tests for syphilis are not immunologically specific. Their specificity is, rather, an empirical one based on a high degree of association. Basically, therefore, aberrant reactions or departures from the association are not truly false, only false from the point of view of syphilology. They are, nevertheless, of considerable practical importance.

False negative reactions are in part a technical matter; serologic tests in general use give 80 to 90 per cent positive reactions with known sera and any test which gives more than 80 per cent positives is regarded as satisfactory. The presence of insufficient reagin accounts for many false negative reactions and occurs in several stages of the disease, especially in early primary syphilis (a positive reaction usually does not develop until two to three weeks after infection) and late syphilis which is latent or localized. Tabes, for example, gives about 40 per cent negative reactions.

False positive reactions may be a result of technical error or may be biological in nature. A certain proportion of non-syphilitic individuals suffering from other infections give positive serologic tests for syphilis. All cases of yaws are Wassermann-positive, of course, and it is reported that 4 to 10 per cent of cases of malaria are positive. Febrile disease of one sort or another occasionally produces isolated or repeated positive reactions. The nature of the false positive reaction in non-febrile persons has been of very considerable interest. Neurath and his co-workers[49] have found that syphilitic and false positive sera differ from one another in several ways, including an inhibition of the complement-fixation reaction by the addition of crude human serum albumin in the case of false positive serum but not with syphilitic serum, and the absorption of the antibody activity of syphilitic sera but not of false positive sera on calcium phosphate. In the false positives produced by unrelated febrile disease these differences do not hold true. In general, however, false positive reactions do not occur with sufficient frequency to detract seriously from the diagnostic value of the serological tests.

The nature of the response to infection with the spirochete that results in the appearance of reagin is not clear. Its lack of immunological specificity would seem to rule out the usual type of antibody response, but if it is to be regarded as antibody, the facile disappearance of reagin during therapy rather than seroresistant syphilis requires explanation. It is possible that reagin represents iso-antibody that is detectable only because it is being produced more rapidly than it can be removed by union with antigen. Phenomena that are possibly analogous have been observed in other diseases. Hughes[50] has shown that there is a precipitin present in convalescent yellow fever serum which reacts with serum taken during the acute stage of the disease but not with yellow fever virus; the precipitinogen has been regarded by some as a "pathologic protein." The observation of Avery, Abernathy and McLeod[51] is similar but reversed as regards specificity; they found that a substance appeared in the blood of human beings and monkeys during the course of various etio-

[49] Neurath *et al.*, preliminary report Science, 1945, *101*:68; and a series of six papers, *cf.* Putnam, Volkin, Craig and Neurath: Amer. Jour. Syph., 1947, 31:457.
[50] Hughes: Jour. Immunol., 1933, 25:275.
[51] Avery, Abernathy and McLeod: Jour. Exp. Med., 1941, 73:173.

logically unrelated infections that specifically precipitated the somatic poly-
saccharide, or C substance, of pneumococci. In general, however, the diagnos-
tic serologic tests for syphilis have contributed little to an understanding of
immunity to this disease.

Pathogenicity for Lower Animals. Syphilis may be transmitted to
anthropoid apes, such as the chimpanzee and the gibbon, and, with less cer-
tainty, to monkeys. Scarification of the genitals or eyebrow results in the de-
velopment of a primary chancre followed in a few weeks by the appearance
of the lesions of secondary syphilis. Subcutaneous, intraperitoneal or intra-
venous inoculation is ineffective.

Rabbits may be infected by inoculation of the anterior chamber of the eye.
The local wound heals, but in four to six weeks a pericorneal congestion de-
velops which is followed by pannus and keratitis; then retrogression and heal-
ing take place. The entire process may take many weeks to complete. Intra-
testicular inoculation produces an orchitis and intrascrotal inoculation a pri-
mary chancre; generalized lesions characteristic of secondary syphilis follow.
A generalized infection may be produced by the intravenous inoculation of
very young rabbits.

In rats and mice no symptoms follow inoculation but the spirochetes mul-
tiply in the tissues and the infection remains latent indefinitely. Guinea pigs
react in much the same way, though a local reaction may develop following
intracutaneous inoculation in the perineal fold.

YAWS (TREPONEMA PERTENUE) AND BEJEL

Two treponemiases, closely related to and perhaps varieties of syphilis, may
be differentiated from it and from one another on the basis of geographical
distribution, method of transmission and certain clinical aspects of the disease.
These are yaws or *frambesia tropica* and bejel or desert syphilis.

Yaws. Yaws is a disease occurring in tropical countries and is common
in Equatorial Africa, the tropical regions of the Far East though rare in India,
the West Indies and tropical America. The causative agent is *Treponema per-
tenue* which was described by Castellani in 1905.

The spirochete is found in the serous exudate of the cutaneous lesions and
in the lymphatics. It may be demonstrated in Giemsa-stained smears or by dark-
field examination of fresh preparations, and is morphologically indistinguish-
able from *T. pallidum.*

The disease in man is characterized by a papular eruption. A general malaise
precedes the appearance of the initial lesion which is practically always extra-
genital and takes the form of a single papule or a small group of them. The
papule enlarges to a diameter of 3 to 4 mm., the thickened epidermis cracks
and the fungoid mass beneath exudes a seropurulent fluid. The mass enlarges
to 3 to 5 cm. in diameter and the lesion is termed a yaw. It eventually dries,
leaving only a scar. Six weeks to three months after the primary lesion appears,
a secondary eruption occurs which is similarly preceded by a general malaise.
The lesions are of the same general character as the primary lesion, appearing
on the extremities, neck and at the juncture of the skin and mucous mem-
brane about the nose, mouth and anus. Tertiary lesions similar to those of
syphilis are said to be rare, but recent work suggests that they may be more

common than has been supposed. The Wassermann reaction is positive and
in certain stages, as in late secondary eruption, differential diagnosis may be
very difficult.[52]

Yaws may be produced by the inoculation of monkeys and rabbits. The
infection in rabbits with *T. pertenue* is similar to that with *T. pallidum* but
relatively few spirochetes are found in the lesions, multiplication is arrested
early, and the inflammatory reaction is slight.[53]

The disease is not venereal and occurs predominantly in children. The spi-
rochete is unable to penetrate the intact skin and enters via abrasions after
deposition either by contact or by insects. Kumm[54] has found that in Jamaica
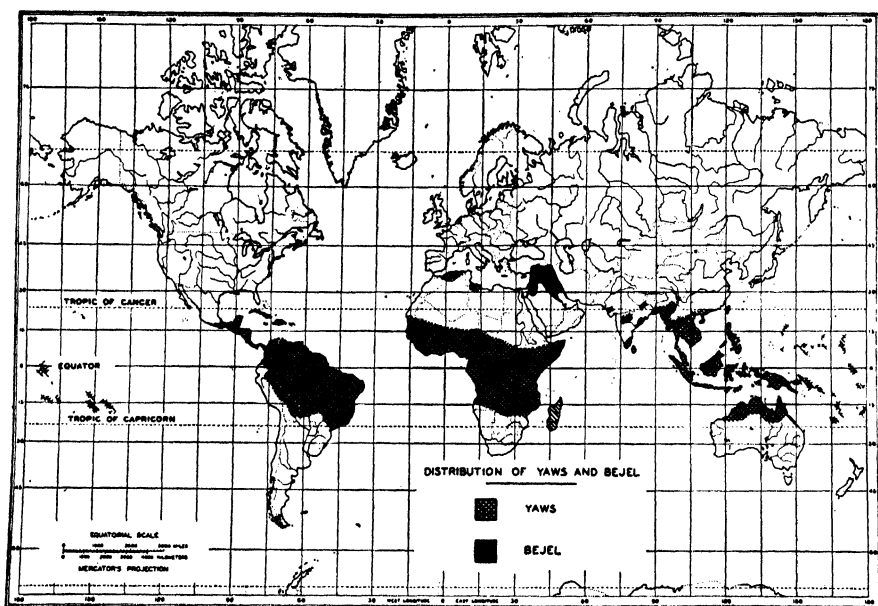
Fig. 201. The world-wide distribution of yaws and bejel. Redrawn from map prepared by
Army Medical Intelligence, 1943. Based on Goode *Base Map* No. 201M. By permission
of the University of Chicago Press.

the small fly, *Hippelates pallipes,* feeds upon the serous exudate in huge num-
bers, the spirochete surviving for some hours in the diverticulum of the fly and
possibly being regurgitated when the fly feeds upon a skin abrasion.

The relationship of yaws to syphilis is very close, the points of difference
being the non-venereal transmission of yaws, the differences between the yaw
and the indurated hunterian chancre, and the relative infrequency of visceral
and tertiary lesions. Yaws is regarded by many as a tropical type of syphilis,
modified by continuous residence in the black races in tropical climates.[55]

[52] *Cf.* Turner, Saunders and Johnston: Report of the Jamaica Yaws Commission, 1932.
[53] Ferris and Turner: Arch. Path., 1938, 26:491.
[54] Kumm: Report of the Jamaica Yaws Commission, 1935.
[55] See Chambers: Trans. Roy. Soc. Trop. Med. Hyg., 1937, *31*:245; Turner: Amer.
Jour. Hyg., 1937, 25:477.

Bejel. A disease of desert Arabs, called by them bejel, has been reported by Hudson.[56] Like yaws it is non-venereal and occurs predominantly in children. It differs from yaws, however, in that mucous patches in the mouth occur with considerable frequency while they are uncommon in yaws. Nervous manifestations are said to be rare but Hoff and Shaby[57] have reported that meningovascular syphilis occurs with some frequency in Bedouin Arabs, if this may be called bejel. The disease has not been studied at length but appears to be very closely related to, if not identical with, syphilis, differing only in its non-venereal transmission. Butler[58] regards it as congenital syphilis. In this connection it is of interest that an outbreak of non-venereal syphilis in children and adult women, perhaps bejel, has been reported in India.[59] Hudson[60] is of the opinion that treponematosis is basically the same, in that yaws represents a non-venereal treponematosis and syphilis a venereal form developed in temperate climates partly under the influence of improved economic and sanitary conditions.

PINTA (TREPONEMA HERREJONI)[61]

Pinta, known in Mexico as *mal de pinto* and in Colombia as *carate,* is clinically a skin disease in which dyschromic changes occur in patches which may be discrete and small or large and confluent, and of a grey, bluish grey, or pinkish color and eventually white. It is especially prevalent in Mexico where 11 per cent of over two million persons examined had the disease, and in Colombia where as much as 4 per cent of the population in some districts are affected. It also occurs in Cuba and the West Indian Islands, and in Central America and tropical South America. So far as is known it is confined to the Western Hemisphere.

Etiology. For many years this disease has been of uncertain etiology though generally assumed to be a fungous infection. It was suggested by Herrejon in 1927 that the disease is caused by a spirochete but it was not until 1938 that a spirochete was first demonstrated by Armenteros and Triana in Cuba. The observation has been amply confirmed and it is now established that pinta is caused by a spirochete which has been named *Treponema herrejoni* by the Cuban workers and *Treponema carateum* by Brumpt.

The spirochete may be found in material taken from early cutaneous lesions and in that aspirated from lymph glands. It stains with Giemsa and by the usual silver impregnation methods used for *T. pallidum,* and may be found by darkfield examination of fresh material. Morphologically it is very similar to, perhaps indistinguishable from, the spirochete of syphilis. Attempts to cultivate it have failed, but it has been reported that the rabbit may be infected.[62] The experimental disease in human volunteers has been studied in considerable detail, however, especially by Leon y Blanco.

[56] Hudson: Arch. Dermat. Syph., 1936, 33:994; Amer. Jour. Trop. Med., 1938, 18:675.
[57] Hoff and Shaby: Trans. Roy. Soc. Trop. Med. Hyg., 1940, 33:549.
[58] Butler: Amer. Jour. Clin. Path., 1939, 9:1.
[59] Iswariah and Nair: Jour. Indian Med. Assn., 1938, 7:651.
[60] Hudson: Amer. Jour. Trop. Med., 1946, 26:135.
[61] Summarized by Holcomb: U. S. Naval Med. Bull., 1942, 40:517.
[62] Leon y Blanco and Oteiza: Science, 1945, 101:309.

The Disease in Man.[63] In the experimental infection the incubation period is from seven to twenty days, the initial lesion being a papule which appears at the site of inoculation. This papule spreads peripherally to form a squamous, erythematous patch reaching a diameter of 1 cm. in four or five weeks. It continues to spread, varying considerably in appearance, and may be lichenoid or psoriaform. Blanco regards this as the primary stage. In about five months the secondary stage begins with the appearance of secondary lesions about the initial lesion and elsewhere on the body. Progressive hyperpigmentation occurs and depigmentation, resulting in varied hues, follows in the third stage; keratosis and superficial atrophoderma become apparent. Hyperkeratosis on the plantar surfaces occurs with some frequency in Cuba but is seldom observed in Mexico. The Wassermann reaction is almost always negative in the primary stage, positive in something over half the cases in the secondary stage, and practically always positive in the tertiary stage. Correspondingly, superinfection is readily produced in the first stage but not in the third stage. Syphilitic individuals may be infected without difficulty.

Transmission. Experimental human infections have shown that serous fluid from the early lesion is highly infectious when placed on an abraded area on the skin, and the spirochete may be demonstrated in the discharge from the fissures in plantar hyperkeratosis. Contact infection would, therefore, appear to be possible but general opinion is that it is not an important method of spread. Simulium flies have been suspected of transmitting the disease, but as yet an insect vector has not been demonstrated.

OTHER TREPONEMAS

A number of other species of Treponema are known. *T. refringens* is found about the genitals and may be confused with the spirochete of syphilis. Others, *T. microdentium, T. macrodentium* and *T. mucosum,* are found in the mouth and apparently are non-pathogenic. A saprophytic water form, *T. elusum,* has been described.

Another species, *T. cuniculi,* is morphologically very similar to *T. pallidum* and is responsible for a venereal disease of rabbits known as rabbit syphilis and sometimes called pallidoidosis. The lesions are small, scaly patches found about the perineal region or on the genitals.[64]

LEPTOSPIRA

(Weil's Disease or Infectious Jaundice; Swamp Fever; Japanese Seven-Day Fever)

Leptospirosis, or infection with leptospira, is a not uncommon and widely distributed affection. Several species of leptospira are involved and the infections differ somewhat in their clinical manifestations.

Morphology. The leptospiras differ from Treponema and Borrelia species in that their spirals are very fine and close, one or both ends of the cell may be hooked, and the individual cells are smaller, not more than 0.3 μ in breadth and generally 6 to 10 μ in length, although exceptionally forms as long as 25 to 30 μ may be observed. The protoplasm is spirally wound about a delicate but firm, elastic, smooth axial filament. Extruding granules, found in *T. pallidum,*

[63] See Lieberthal: Jour. Amer. Med. Assn., 1943, 123:619.
[64] See studies of McLeod and Turner: Amer. Jour. Syph., 1946, 30:442, 455.

THE LEPTOSPIROSES

Leptospira		Disease in Man	Animal Reservoirs		Infections in Other Animals	Geographical Distribution
Proper Name	Synonyms		Rats	Mice		
Lept. icterohemorrhagiae	"Wijnberg"	Weil's disease	*R. norvegicus*, *R. rattus*	....	Dog, fox, cat, horse, cattle	World-wide, especially Europe and the Americas
Lept. grippo-typhosa	Andaman Sumatra 70, "Moskou V"	Swamp fever, Andaman fever	....	*Microtus arvalis*, *Apodemus sylvaticus*, *Evotomys glareolus*	Dog, horse, cattle	Europe, N. E. I.
Lept. sejroe	....	Swamp fever, infectious jaundice	"Rats" (Italy)	*Apodemus sylvaticus*, *Mus spicilegus*	....	Denmark, Italy
Lept. canicola	"Roesel"	Infectious jaundice	....	....	Stuttgart disease in dogs	World-wide
Lept. hebdomadis	....	Japanese seven-day fever, nanukayami	....	*Microtus montebelli*	Dog	Japan
Lept. autumnalis	Akiyami A, Rachmat	Hasami fever	"Field rat"	"Field mouse", *Apodemus speciosus*	....	Japan, Sumatra
Lept. bataviae	*Lept. javanica*	Infectious jaundice	*R. norvegicus*, *R. decumanus* (Japan), "Rats" (Italy)	*Apodemus sylvaticus*, *Micromys minutus*, *Micromys soricinus*	Cat	N. E. I., Japan, Italy
Lept. pyrogenes	Salinem	Infectious jaundice	*R. brevicaudatus*, *R. rattus*		Cat	N. E. I.
Lept. australis A	....	Mossman fever	*R. rattus*, *R. culmorum*	....	....	Australia
Lept. australis B	....	Mossman fever	*R. rattus*, *R. culmorum*	....	....	Australia
Lept. pomona	*Lept. mezzana*	Pomona fever, swamp fever, swineherds' disease	"Rats" (Italy)	....	Pig (Switzerland)	Australia, Italy, Switzerland

are not present even in electron micrographs.[65] Like the finer bacterial flagella, the axial filament is not seen in the darkfield or ordinary stained preparations but may be demonstrated by silver impregnation flagella stains. The periplast is thick and almost transparent and appears in the darkfield as a narrow, clear zone or halo and in certain stained preparations as a grayish or unstained halo. The mechanism of locomotion is more complex than in the treponemas; the hooked ends of the axial filament are probably involved, and when the bow-like axial filament is alternately straightened and relaxed by rhythmic contractions of the protoplasmic spirals the cell rotates.

The leptospiras are readily filterable through Berkefeld V and N candles because of their small breadth and active motility, and filtration is sometimes used to separate them from bacteria. Treponemas are usually not filterable by ordinary suction methods but grow through the pores of Berkfeld candles with some facility.

Cultivation. The leptospiras are the most readily cultivable of the spirochetes and may be grown on serum diluted with 5 or 10 parts of Ringer or Locke solution. A relatively simple and reproducible serum medium has been described by Stuart.[66] Growth is considerably better in a semisolid medium and Noguchi[1] has used a mixture of 1 part of 2 per cent nutrient agar, 1 part of rabbit serum and 8 parts of physiological salt solution. They have not been cultivated in synthetic media but Rosenfeld and Greene[67] have found that nicotinic acid and amide, thiamine, and riboflavin markedly stimulate growth. In the isolation of leptospira from water, agar medium and blood may be added to Berkefeld V and N filtrates. According to Schüffner the guinea pig may be used as a "living filter"; if leptospira contaminated with other bacteria are inoculated intraperitoneally, they enter the circulation much more rapidly than the contaminants and may be found in heart's blood in pure culture ten minutes after inoculation. The leptospiras are all obligate aerobes. Those tested do not ferment sugars and are indifferent to the presence of carbohydrates in the medium.

Pathogenicity. The spirochetes grouped in the genus Leptospira are immunologically related but distinguished from one another by agglutination determined by microscopic examination under the darkfield.[68] Unlike Borrelia, they are not predominantly blood parasites but are also found in the tissues, especially the kidney, and are commonly excreted in the urine. The infections they produce in man are all similar and characterized by a febrile reaction and the occurrence of jaundice to a greater or lesser extent. Of these Weil's disease or infectious jaundice is perhaps the best known since it has occurred in Europe for many years. A related infection is found in Central and Eastern Europe and in Italy, and still others occur in the South Pacific region, in the East Indies, and Australia, and also in Japan. These diseases have been reviewed by Walch-Sorgdrager[69] and are summarized in the accompanying table. The reservoir of infection is rodents, especially rats and various kinds of

[65] Morton and Anderson: Jour. Bact., 1943, 45:143.
[66] Stuart: Jour. Path. Bact., 1946, 58:343.
[67] Rosenfeld and Greene: Jour. Bact., 1941, 42:165; Greene: Jour. Bact., 1945, 50:39.
[68] Cf. Gardner: Lancet, 1947, i:20.
[69] Walch-Sorgdrager: Bull. Health Organization, (League of Nations), 1939, 8:143.

mice, and more recently the dog has been found to be of some importance. Human infection is probably almost always acquired from animals, usually by the contamination of water with infected urine.

Weil's Disease (Infectious Jaundice).[70] The causative agent of Weil's disease, *Leptospira icterohemorrhagiae*, was discovered in 1914 by Inada and Ito in Japan and was found in Germany the following year by Hübener and Reiter and by Uhlenhuth and Fromme. It was called *Spirochaeta icterogenes* by the German workers. The disease has been found all over the world; it is very common in Japan, less so in Europe where the greatest incidence is in the Netherlands and France, and is not uncommon in South America. It is probably not so rare in the United States as has been supposed.[71]

Disease in Man. The incubation period is six to twelve days, and the high initial fever is followed by nausea and vomiting, epistaxis, headache and muscular pains, and there may be a moderately severe bronchitis. Jaundice is not always observed; it occurred in 40 to 60 per cent of the Dutch cases. Convalescence is slow and weakness may persist for months.

The leptospira are distributed throughout the body in the first week of illness and may be demonstrated in the blood by guinea pig inoculation but rarely in blood smears. After the first week they are present in the urine and may continue to be so excreted for four to five weeks. There is some evidence that the case fatality is reduced by treatment with antiserum. In fatal cases death usually occurs during the second or third week, but occasionally as early as the end of the first week. The case fatality is variable; it has varied from 4.6 to 32 per cent in Japan and has been about 10 per cent in the Dutch cases and as high as 25 per cent in Scotland. At autopsy the leptospira are found in almost all the organs and tissues in those dying during the initial febrile stage, but if death occurs in the second week or later, they are rarely found elsewhere than in the kidneys.

Immunity. Recovery from the disease is accompanied by the development of a solid immunity, and a specific lysin is present in the blood which persists in detectable amounts for several years. Agglutinins appear in the convalescent stage, sometimes to very high titer such as 1 : 100,000, but titers of 1 : 400 are significant. The agglutination test has considerable diagnostic utility in both man and animals.[72] Prophylactic inoculation has given encouraging results.

Transmission. The wild rat is the most important animal reservoir of infection and Schüffner[73] has reported that 40 per cent of tame rats raised for experimental purposes have been found to be infected in Holland. The infection may be detected by darkfield examination of urine and kidney emulsion, and preferably by the inoculation of young guinea pigs with kidney emulsion. Larson[74] has found that a fatal infection may be produced in young mice, three to four weeks old.

[70] The literature to 1941 has been reviewed by Ashe, Pratt-Thomas and Kumpe: Medicine, 1941, 20:145.

[71] See, for example, the experience recorded by Bruno, Wilen and Snavely: Jour. Amer. Med. Assn., 1943, 123:519.

[72] For the development of a macroscopic test see Starbuck and Ward: Jour. Inf. Dis., 1942, 70:88.

[73] Schüffner: Ztschr. f. Immunitätsf., 1941, 99:323.

[74] Larson: Pub. Health Repts., 1941, 56:1546; *ibid.*, 1943, 58:10.

The proportion of infected rats is variable from one locality to another; in New York about 4 per cent of the rats have been found to be infected, in Japan 40 per cent, in England 30 per cent, in Rotterdam 7 to 40 per cent and in Philadelphia about 10 per cent. The leptospira are discharged in the urine of infected rats and are transmitted to man via stagnant water contaminated with rat urine. Weil's disease is to some extent an occupational disease and occurs in coal miners, sewer workers and others working in contact with contaminated water.[75] In the first World War there were many cases in troops stationed in trenches containing stagnant water. The majority of the Dutch cases have been in swimmers, bargemen and fishermen and those who by accident or intent fall into the canals. In one instance a water-borne epidemic occurred in which the water was presumably contaminated by rats from a neighboring sewer.[76] The manner in which the spirochetes enter the body is not known, but very possibly it is through minute cuts and abrasions in the skin or via the alimentary tract.

Canine Leptospirosis. The role of dogs in the transmission of infectious jaundice has been of considerable interest. Dogs suffer from two forms of leptospirosis, the one an acute jaundice ("yellows") similar to acute Weil's disease in man, and the other a non-jaundice type known as Stuttgart disease or canine typhus. *Lept. icterohemorrhagiae* may infect dogs and probably is responsible for the former type of disease, while a canine leptospira, *Lept. canicola,* produces the latter form. The relative incidence of the two infections in dogs is not known with certainty; some have suggested that as high as 50 per cent of canine leptospiroses are *icterohemorrhagiae* infections. *Lept. canicola* infection in dogs appears to be relatively common in the United States. Raven[77] found 38 per cent of rural dogs and 28 per cent of urban dogs examined in Pennsylvania showed serological evidence of infection; and Greene[78] found about 20 per cent of dogs selected at random and 29 per cent of dogs from hospitals in California showed the presence of antibody. Man may acquire Weil's disease from dogs and a number of human infections with *Lept. canicola* have been reported since 1934 in the Netherlands and more recently from this country.[79]

Another species of leptospira, *Lept. sejroe,* has been described as the cause of human infection in Denmark and in Italy. The cases showed milder symptoms than those infected with *Lept. icterohemorrhagiae.*

Swamp Fever. A leptospiral infection prevalent during the summer and early autumn months in Bavaria, Silesia and in the Volga region is variously known as swamp fever, *Schlammfieber,* autumn fever, water fever, field fever, harvest fever, mud fever and slime fever. It has also been reported from Holland. It is found among field workers following floods or in swampy districts particularly during the hay harvest. Clinically it closely resembles Weil's disease in a mild form—the case fatality is less than 1 per cent—but there is no jaundice except occasionally in the sclera. The causative microorganism, *Lept.*

[75] The epidemiology of leptospirosis is discussed by Molner and Kasper: Amer. Jour. Pub. Health, 1941, 31:945.
[76] Jorge: Bull. Off. Int. Hyg. Pub., 1932, 24:88.
[77] Raven: Jour. Inf. Dis., 1941, 69:131.
[78] Greene: Amer. Jour. Hyg., Sec. B, 1941, 34:87.
[79] See the discussion by Rosenbaum: Arch. Int. Med., 1946, 78:531.

grippo-typhosa (*Spirochaeta grippo-typhosa*), is immunologically distinct from *Lept. icterohemorrhagiae*. The reservoir of infection is the field mouse, *Microtus arvalis;* the wood mouse, *Apodemus sylvaticus,* and the bank-vole, *Evotomys glareolus,* are less often infected. This disease has been of particular interest in recent years and has been reviewed by a number of workers.[80] A very similar disease is found in the rice fields in Italy but the etiology appears to be diverse in that a number of species of leptospira have been found, in many instances apparently identical with those found in the Far East. A similar situation holds true for *swineherds' disease* (Bouchet-Gsell disease) found in Switzerland; pigs are infected with *Lept. pomona,* also found in Australia, and are the source of human infection.[81] Infection of cattle and man with a leptospira closely related to but not identical with *Lept. grippo-typhosa* has been reported[82] from the Near East, and possibly the human infection may be contracted from cattle.

Leptospira Infections of the Far East. A number of leptospira infections occur in the Far East similar to swamp fever in that they are febrile, jaundice is rare except in the sclera, and the case fatality rate is low. *Japanese seven-day fever,* or *nanukayami,* is prevalent in Japan in the autumn. The causative agent is *Lept. hebdomadis* and is carried by the field mouse, *Microtus montebelli.* A common sequel to the infection is opacification of the vitreous humor. *Hasami fever* is a similar disease also prevalent in Japan and caused by *Lept. autumnalis* (*Lept. akiyami A*). The reservoir of infection is the mouse *Apodemus speciosus* and possibly other species of field mice and rats are infected.

Leptospirosis of heterogeneous etiology is not uncommon in the Netherlands East Indies. The disease occurring in Sumatra known as *Rachmat infection* is caused by *Lept. autumnalis* and is presumably identical with Hasami fever in Japan. Other infections are known by various names, including *Andaman A fever, Salinem infection* and the like. The causative organism of Andaman fever appears to be *Lept. grippo-typhosa.* The name *Lept. pyrogenes* has been given the causal agent of Salinem fever. The remainder of the leptospiroses appear to be largely infections with *Lept. bataviae* which is found in natural infections in rats, including *Rattus norvegicus* and *R. decumanus* (in Japan), and in field mice including *Apodemus sylvaticus, Micromys minutus* and *M. soricinus.*

Mild fevers of leptospiral etiology are endemic in Queensland in Australia, and a number of immunologically distinct varieties of leptospira have been isolated. That designated *Lept. pomona* is the causative agent of the affection known as *Pomona fever,* and other species named *Lept. australis A* and *Lept. australis B* are responsible for febrile disease designated *Mossman fever, coastal fever* and the like. Additional varieties have also been reported but it is not as yet clear that they are distinct species.

Geographical separation of the leptospiroses is not altogether sound for at least some of these microorganisms occur in widely separated regions. Thus

[80] Kathe: Med. Klin., 1941, 37:892; Klin. Woch., 1942, 21:791; Ztschr. f. Immunitätsf., 1943, 103:60; Uhlenhuth: Ztschr. f. Immunitätsf., 1943, 103:35; Schüffner: Klin. Woch., 1942, 21:787.
[81] See Gsell: Schweiz. Med. Woch., 1946, 76:237.
[82] Bernkopf, Olitski and Stuczynski: Jour. Inf. Dis., 1947, 80:53; *ibid.,* 1948, 83:232.

Lept. grippo-typhosa is found in the Netherlands East Indies and *Lept. bataviae* and *Lept. pomona* have been reported as the etiologic agents of swamp fever in Italy. There is as yet no evidence that leptospira other than *Lept. ictero-hemorrhagiae* and *Lept. canicola* occur in the United States.

Saprophytic Leptospira. Leptospira closely resembling *Lept. ictero-hemorrhagiae* have been isolated from water by a number of workers. These non-pathogenic forms have been termed *Lept. biflexa* in this country and in England, and *Spirochaeta pseudoicterogenes* by the German workers. Their relation to the pathogenic leptospira is uncertain; Uhlenhuth and Zuelzer[83] have reported observations which suggested that the water leptospira are avirulent forms of *Lept. icterohemorrhagiae* whose virulence may be restored by animal passage. These results have not been confirmed by other workers.

RAT-BITE FEVER (SPIRILLUM MORSUS MURIS)

There are two distinct kinds of disease which may follow the bite of rats and are designated rat-bite fever. One, an actinomycete infection, is discussed
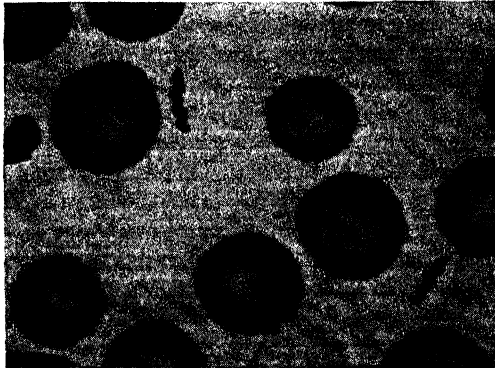
Fig. 202. *Spirillum morsus muris* in blood of an inoculated mouse (Van Sant).

elsewhere (p. 670). The other, known in Japan as *sodoku*, is caused by a spiral microorganism discovered by Futaki and his co-workers[84] in 1916. Following the bite of an infected rat, the original wound heals but, after an incubation period of ten to twenty-two days, becomes inflamed and painful. Fever, swelling of the lymph glands, skin eruptions and other symptoms occur. The fever is of the relapsing type, with paroxysms at fairly regular intervals, usually about once a week, which continue to recur for one to three months or longer. The case fatality varies from 2 to 10 per cent.

The characteristic spiral microorganism has been found in the swollen local lesions of the skin and the enlarged lymph glands and also in two instances in the circulating blood. Guinea pigs and mice may be infected by the inoculation of blood or fluid expressed from the local lesions. The microorganism is found in about 3 per cent of the house rats in Japan. The rat is not the only vector of the infection, for cases of what is apparently the same disease have been

[83] Uhlenhuth and Zuelzer: Klin. Wchnschr., 1922, 1:2124.
[84] Futaki, Takaki, Taniguchi and Osumi: Jour. Exp. Med., 1916, 23:249.

traced to the bite of the cat, dog, pig, ferret, squirrel and weasel. Ripley and Van Sant[85] have reported the case of a medical student who acquired the infection from a cut during an operation on a dog.

The disease has long been known in Japan and has been reported from many localities all over the world. Brown and Nunemaker[86] have found a total of 125 cases reported in the United States from 1916 to the end of 1940. What proportion of the earlier reported cases were actinomycete infections is, of course, problematical. Of 40 cases reported in 1931–1940, the spirillum was demonstrated by animal inoculation in 17; of these 11 resulted from rat bite and the remainder had a history of mouse bite, cat bite or scratch, contact with dogs, or trauma without known animal contact. The natural occurrence of spirochetes in laboratory mice, rats and guinea pigs is a source of error to be guarded against in laboratory diagnosis by animal inoculation. There is little doubt that the affection is much more common than appears from the records, for the cases occur sporadically and their true nature has often gone unrecognized. The infection has been produced in man by artificial inoculation for the treatment of paresis; according to Brown and Nunemaker[86] there are 104 such cases in the English literature.

Not a great deal is known of the immune response to the infection other than that a spirillicidal antibody is produced that is also responsible for immobilization of the spirilla in immune serum. Savoor and Lewthwaite[87] have observed that a positive Weil-Felix reaction of the OXK type (p. 821) is produced in experimental animals but the antigen shared with these strains of Proteus is distinct from that which is responsible for the spirillicidal antibody.

The spiral microorganism of rat-bite fever has been variously classified as *Spirochaeta morsus muris*, *Borrelia muris*, *Spirillum minus*, etc. It is shorter— 2 to 5 μ in length—than the recognized spirochetes, is relatively rigid and possesses polar tufts of flagella which give it a rapid darting motion unlike the flexible undulating movements of the spirochetes. The clinical symptoms of the disease are typical of spirochetosis, however, and the arsenicals are effective therapeutic agents. Originally given the name Spirochaeta, this microorganism was found[88] to be identical with *Spirillum minus* found by Carter[89] in the blood of a rat in India. It is, perhaps, best given the independent generic name Spirillum[90] and considered with the spirochetes.

[85] Ripley and Van Sant: Jour. Amer. Med. Assn., 1934, 102:1917.
[86] Brown and Nunemaker: Bull. Johns Hopkins Hosp., 1942, 70:201.
[87] Savoor and Lewthwaite: Brit. Jour. Exp. Path., 1941, 22:274.
[88] Robertson: Ann. Trop. Med. and Parasit., 1930, 24:367.
[89] Cited by Robertson.
[90] Dubosq and Lebailly: Compt. Rend. Acad. Sci., 1912, 154:535.

Chapter 34

MEDICAL PARASITOLOGY

By Richard J. Porter, Ph.D.*

Medical parasitology concerns those organisms parasitic in or on man which belong to the animal kingdom. The most important species belong to four phyla of animals, the *Protozoa*, or single-celled animals, the *Platyhelminthes*, or flatworms, the *Nemathelminthes*, or roundworms, and the *Arthropoda*. The arthropoda, which include crustacea, insects, mites, spiders, scorpions, etc., are of medical importance primarily as agents of disease transmission and are therefore considered here only insofar as they affect the epidemiology of infections caused by other agents.

Animal parasite infections act fundamentally like infections with other agents. For several reasons, however, the general methods of study differ from those utilized for other infectious organisms. The larger size of animal parasites and their greater visible complexity of structure make morphology a particularly valuable tool in identification. This is especially important because few can be readily cultivated, and physiological bases for their recognition are therefore not generally available. The life-cycles of these organisms are in some cases exceedingly complex, and the elaboration of the complete reproductive histories of many animal parasites has dominated their study. These considerations have obscured the fundamental similarities between infections with animal and plant parasites. It should be emphasized that the differences between diseases caused by different animal parasites are often more significant than the differences between such diseases and those caused by bacteria or other agents. Thus, amebic dysentery has more medical features in common with bacterial dysentery than with the protozoan disease, malaria. Because of the great variety among animal parasites, the discussion in the following pages is centered, wherever possible, about representative organisms. It should be borne in mind that many important species will thereby receive less space than they deserve. For fuller discussions of these parasites the reader is referred to the standard texts of parasitology.[1]

THE PROTOZOA

The Protozoa are animals which carry on all the functions of life in a single cell. They are generally considered analogous to the individual cells composing the higher animals or Metazoa, though they are often capable of independent existence under a wider variety of conditions than are metazoan cells. Many thousand species of protozoa have been described, though less than thirty well-defined species are known to parasitize man. They constitute an exceedingly heterogeneous group of organisms, varying in size from that of the larger bacteria to several centimeters in diameter, in complexity of structure from a simple, formless cell like *Endamoeba histolytica* to organisms of far greater intricacy than many metazoa, and in life-cycle from the simple binary fission of *Trichomonas vaginalis* to the alternation of hosts and of asexual and sexual reproduction exhibited by the malarial parasites. The following classification, adapted from Wenyon,[2] suggests the diversity of this group:

Sub-phylum Plasmodroma. Movement by thin whip-like filaments called flagella, or by temporary protoplasmic extrusions, pseudopodia.
> Class *Rhizopoda*. Movement entirely or predominantly by pseudopodia. Ex.: *Endamoeba histolytica* of human dysentery.
> Class *Mastigophora*. Movement at least predominantly by flagella. Ex.: *Trypanosoma gambiense* of African sleeping sickness.
> Class *Cnidosporidia*. These produce characteristic spores provided with extrusible filaments. All are parasitic in invertebrates or cold-blooded vertebrates. Ex.: *Nosema bombycis*, agent of pébrine disease of silkworms, celebrated by the researches of Pasteur.
> Class *Sporozoa*. Reproduction typically by multiple fission (schizogony) alternating with sexual reproduction (sporogony) which gives rise to sporozoites, the infective stage. Alternation of hosts is common. All are parasitic. Ex.: *Plasmodium* species of human malaria.
Sub-phylum Ciliophora. Movement by numerous short fine protoplasmic processes, cilia. Most have two types of nuclei. Ex.: *Balantidium coli* of human dysentery.

For material supplementing that presented in the following pages, the reader is referred to the textbooks of protozoology.[2]

THE INTESTINAL AMEBAE (RHIZOPODA)

Endamoeba Histolytica.[3] Lewis[4] in 1870 and Cunningham[5] in 1871 first reported amebae, probably the non-pathogenic *Endamoeba coli*, in human feces. Lösch[6] in 1875 described what were apparently *E. histolytica* in the stools and intestinal ulcers of a fatal case of dysentery. He found similar ulcers containing the amebae in an artificially infected dog. They were first ob-

---

[2] Wenyon: *Protozoology*. Williams & Wilkins Company, Baltimore. 1926; Hegner and Taliaferro: *Human Protozoology*. The Macmillan Co., New York. 1924; Thomson and Robertson: *Protozoology*. Williams & Wilkins Company, Baltimore. 1929; Craig: *Laboratory Diagnosis of Protozoan Diseases*. Lea & Febiger, Philadelphia. 1948.

[3] A valuable comprehensive book is Craig: *Etiology, Diagnosis and Treatment of Amebiasis*. Williams & Wilkins Co., Baltimore. 1944.

[4] Lewis: Appendix to Ann. Rept. of Sanitary Commissioner with Govt. of India. Calcutta, 1870.

[5] Cunningham: Ann. Rept. of Sanitary Commissioner with Govt. of India. Calcutta, 1871, pp. 141–243.

[6] Lösch: Arch. path. Anat., 1875, 65:196.

served in liver abscesses by Koch[7] in 1883, and Kruse and Pasquale[8] in 1894 demonstrated their etiological role by producing typical dysentery in cats inoculated rectally with amebae in the bacteriologically sterile pus from a liver abscess.

Characteristics and Life-Cycle. The active ameba as seen in the intestinal ulcers of dysentery cases is a granular, colorless or pale greenish mass of cytoplasm, 15 to 50 (usually 20 to 30) μ in diameter. It has no definite shape. Locomotion is accomplished by the sudden extrusion of clear projections of cytoplasm, the *pseudopodia*, the remainder of the cell body following these pseudopodia in a flowing motion. The granular cytoplasm often contains red blood cells or débris of tissue cells in various stages of digestion. These are the food engulfed by the ameba. The nucleus may be visible as a delicate ring of granules. Reproduction in this stage is by binary fission, the nucleus undergoing a type of mitosis and the cytoplasm then dividing to produce two daughter amebae like the original. In organisms fixed and stained with hematoxylin the nuclear structure is characteristic, consisting of a thin peripheral layer of fine black granules and a central small black dot, the *karyosome* (Fig. 203). The entire nucleus is generally 4 to 5 μ in diameter.

Infected persons with diarrhea or dysentery pass active ameboid parasites in their stools. In the intestinal lumen of a *carrier*, however, the ameba loses its ingested food particles, shrinks to a diameter of 6 to 20 μ, rounds up and becomes essentially non-motile, sending out only an occasional pseudopodium. This is the *precystic stage*, which soon secretes about itself a clear wall, becoming the partially resistant *cyst*. In its passage down the intestine the cyst continues to develop, acquiring a vacuole of glycogen and one or more ovoid rods of black-staining material, the *chromatoid bodies*. The nucleus divides into two and then four, all resembling the nucleus of the active ameboid stage though considerably smaller. In the mature cyst the glycogen vacuole soon disappears, and the chromatoid bodies persist at most for a few days.

The cyst is the infective stage, since it alone can pass through the stomach without destruction. As studied in culture its excystment consists of the emergence of a four-nucleate organism which by a complicated division process produces eight small amebae. These presumably are the stages which enter the tissues of the intestinal wall to initiate a new infection.

The first culture of *E. histolytica* was performed by Boeck and Drbohlav[9] in equal parts of inactivated human serum and Locke's solution tubed over egg slants. Later modifications have substituted other types of serum, Ringer's solution or physiological saline, and different slant media. For short-term cultivation the fluid medium alone is sufficient, and the slant may be omitted.[10] Pure cultures of *E. histolytica* have not been obtained, the amebae growing only in the presence of bacteria. Unlike the amebae in their normal habitat, the colon, the organisms in culture ingest bacteria, yeasts and starch grains. Cysts are formed in certain media containing added rice starch. Some of these cysts

[7] Koch and Gaffky: Arb. a. d. kaiserl. Gesundh., 1887, 3:1.

[8] Kruse and Pasquale: Ztschr. f. Hyg., 1894, 16:1.

[9] Boeck and Drbohlav: Proc. Nat. Acad. Sci., 1925, 2:235; Amer. Jour. Hyg., 1925, 5:371.

[10] Craig: *Laboratory Diagnosis of Protozoan Diseases*. Lea & Febiger, Philadelphia. 1948.

resemble those formed naturally in the intestine, but others are abnormal, containing as many as thirty nuclei.

Amebiasis and Amebic Dysentery. The amebae normally inhabit the mucosa and submucosa of the large intestine.[11] By means of enzymes and mechanical
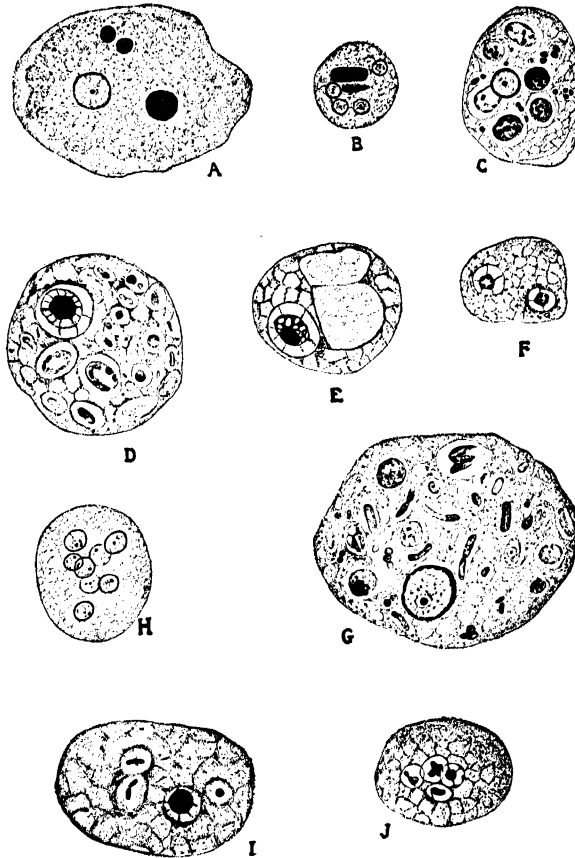
Fig. 203. The amebae living in man. *A.* Active ameboid form of *Endamoeba histolytica* containing three red blood cells. *B,* Mature quadrinucleate cyst of the same containing two chromatoids. *C,* Active ameboid form of *Endamoeba gingivalis.* *D,* Active ameboid form of *Iodamoeba williamsi* containing many intestinal bacteria. *E,* Cyst of same showing large double vacuole which in life was filled with glycogen. *F,* Ameboid form of *Dientamoeba fragilis* containing two nuclei. *G,* Large active ameboid form of *Endamoeba coli* containing intestinal bacteria and débris. *H,* Mature octonucleate cyst of same. *I,* Ameboid form of *Endolimax nana. J,* Mature quadrinucleate cyst of same. *A, B, C, G,* and *H* × about 1300 (Dobell). *D, E, F,* and *J* × 3000 (Taliaferro and Becker). *I* × 3000 (Taliaferro in Hegner and Taliaferro's *Human Protozoology,* courtesy of The Macmillan Company).

action they digest the tissues to produce minute necrotic lesions of the mucosa, which may enlarge into more or less extensive undermined ulcers in the sub-

[11] It is claimed by some that in symptomless carriers the amebae develop in the intestinal lumen rather than in the tissues. *Cf.* Wenyon *et al.:* Trans. Roy. Soc. Trop. Med. & Hyg., 1947, 41:55.

mucosa. The abundance and severity of the lesions determine the clinical picture. The great majority of infected individuals are carriers exhibiting no symptoms. Clinical cases range from moderate diarrhea to acute dysentery with passage of blood and mucus, extreme weakness and, not infrequently, death. A varying proportion, probably between 20 and 50 per cent, of individuals with amebic dysentery shows necrotic abscesses of the liver. These also occur occasionally in individuals who give no history of intestinal symptoms of infection. Lung abscesses, usually produced by extension from the liver through the diaphragm, occur in a small number of individuals, and abscesses have been reported in practically every organ of the body.

It is not yet known why some infected individuals exhibit frank disease while others become carriers without evidence of clinical effects. Walker and Sellards[12] showed that a strain which produces acute dysentery in some individuals may give rise to the symptomless carrier condition in others. This has generally been attributed to differences in natural resistance of infected individuals, but there is evidence suggesting that associated bacteria significantly influence the severity of the amebic lesions.[13] Although the evidence is still inconclusive, it has been claimed repeatedly that some strains of *E. histolytica,* particularly those producing small cysts, are non-pathogenic, or at least much less virulent than others.[14] While the factors in virulence of the infection are unknown, it is clear that the relationship between *E. histolytica* and its host is exceedingly complex.

IMMUNITY. There is no direct evidence of acquired immunity to *Endamoeba histolytica* infection. However, the marked reduction in incidence above middle life suggests that some resistance develops. Natives of hyperendemic regions show acute dysentery less frequently than aliens, possibly as a result of acquired immunity. Serum antibody response to the infection is evidenced by the complement-fixation test, which is positive in over 90 per cent of infected individuals, becoming negative after successful elimination of the amebae by treatment.

DIAGNOSIS. Diagnosis of infection with *E. histolytica* depends upon the finding of characteristic organisms in the stools and their morphological differentiation from the non-pathogenic amebae occurring in human feces (p. 760). Cultivation has been utilized, but in cultures *E. histolytica* resembles the non-pathogenic *E. coli* so closely that differential diagnosis is often very difficult. Direct smears of fresh loose stools in warm saline may reveal the active ameboid stages. They may also be found in material taken directly from lesions in the lower bowel with the aid of the proctoscope. The staining method of Quensel[10] is valuable for specific identification of the ameboid stages. In this technique parasites in fresh stools are stained supravitally with Sudan III and methylene blue. In well-formed stools cysts may be expected, and these are most easily identified in iodine solutions, as D'Antoni's iodine.[10] When cysts are present they may be concentrated by the method of zinc sulfate flotation.[15]

[12] Walker and Sellards: Philippine Jour. Sci. (B), 1913, 8:253.
[13] *Cf.* Deschiens: Ann. Inst. Pasteur, 1938, 61:5.
[14] Wenyon *et al.*: Trans. Roy. Soc. Trop. Med. & Hyg., 1947, 41:55; Faust *et al.*: Amer. Jour. Trop. Med., 1938, 18:169.
[15] Faust *et al.*: Amer. Jour. Trop. Med., 1938, 18:169.

This procedure utilizes the fact that amebic cysts (as well as worm eggs and cysts of other protozoa) rise to the surface of a 33 per cent solution of zinc sulfate (specific gravity 1.180). This method reveals many infections which are missed by direct examination. *E. histolytica* appears irregularly in the stools and, whatever the technique, repeated examinations are often necessary to establish a diagnosis. It should be reemphasized that recovery of the organisms is only the first step in diagnosis of *E. histolytica* infection. The final step is the identification of the parasites, a task requiring abundant experience.

CHEMOTHERAPY. Emetine, a vegetable alkaloid, was long used in the treatment of amebiasis. While it usually has a favorable effect on the symptoms and is of value in amebic liver abscess, it rarely eliminates the infection from the intestine. Several iodine-containing drugs, notably chiniofon and diodoquin, and arsenicals, especially carbarsone, are effective in eradicating the amebae. Nevertheless, some cases are highly refractory to treatment.

Epidemiology and Control. The active ameboid stages of *E. histolytica* die quickly after exit from the body, for they are very susceptible to drying and to changes in temperature and salt concentration. Since they are rapidly destroyed by gastric juice they are not infective when swallowed. The amebic dysentery patient is therefore practically harmless as a source of infection, since only the ameboid stages occur in his stools. The cysts passed by carriers, while not at all comparable to bacterial spores in resistance, show considerably less susceptibility to conditions outside the body than do the ameboid stages. Studies utilizing cysts from culture tested for viability by cultivation show survival of several months in water at 0° C., three days at 30° C., thirty minutes at 45° C., and five minutes at 50° C.[16] Early reports indicated that cysts of *E. histolytica* were unharmed by concentrations of free chlorine over one hundred times as great as those used in water purification. In these tests the organic content of the water is far higher than that encountered in water purification plants. Recent studies indicate that under these conditions the cysts of *E. histolytica* may be no more resistant to chlorine than are such intestinal bacteria as *Bact. coli.*[16] It is not known at present whether this relationship holds under normal water treatment conditions. It is believed that ordinary residual chlorine concentrations cannot destroy amebic cysts but that hyperchlorination is effective. In this connection it should be borne in mind that in the Chicago amebic dysentery outbreak of 1933 there was abundant circumstantial evidence that the infection was spread by water containing sufficient residual chlorine to kill intestinal bacteria.[17]

In general, the spread of *E. histolytica* resembles that of intestinal bacterial infections, utilizing any means by which fecal contamination reaches the human mouth. Most important are drinking water, food-handlers and houseflies. Swimming pools, though not definitely incriminated, are a potential source of infection. Viable cysts have been found in the droppings of houseflies one to two days after exposure, and flies have been held responsible for at least one important outbreak. The 1933 epidemic in Chicago referred to above was traced to local sewage contamination of drinking water in two hotels. This epidemic, in which 1409 cases were discovered and there were 98 deaths, was

[16] *Cf.* Morton: Trop. Dis. Bull., 1948, 45:377.
[17] McCoy *et al.: Epidemic Amebic Dysentery.* Nat. Inst. Health Bull. No. 166. 1936.

the first important outbreak in a city of the temperate zone. It directed medical attention to a problem which had been considered important only in the tropics.

The distribution of *E. histolytica* is world-wide, but temperate regions have usually a low incidence of infection. Surveys indicate a general infection rate in the United States of about 10 per cent, though in some southern localities the incidence has approached 40 per cent. In the tropics the carrier rate is generally very high, often exceeding 50 per cent. Human carriers are the only important source of infection. Although various animals, especially dogs and cats, are susceptible to the infection, and amebae morphologically identical with *E. hisolytica* occur naturally in lower animals, particularly rats, dogs and monkeys, there is no epidemiological evidence that human infections are derived from these amebic infections in lower animals. Furthermore, the evidence that these amebae are *E. histolytica* is not conclusive.

Control of the spread of *E. histolytica* is not significantly different from that of other human enteric infections. The high incidence of carriers not known to have had clinical dysentery complicates the problem, but it is ultimately a matter of prevention of access of human feces to the mouths of susceptibles.

Other Species of Amebae Parasitic in Man. Four other species of amebae live in the human intestine (Fig. 203). Being non-pathogenic they are of medical interest only because they must be ruled out in the diagnosis of *E. histolytica.*

Endamoeba coli, a common species, occurring in 25 to 50 per cent of the general population of the United States, differs from *E. histolytica* in several characters. The stained nucleus shows thicker peripheral chromatin blocks and a larger and usually non-central karyosome. The ameboid stage is sluggishly and usually non-progressively motile with blunt, slowly extruded pseudopodia. It ingests bacteria and other particles but rarely red blood cells. The spherical cysts average somewhat larger, 10 to 33 μ in diameter, contain eight nuclei in the mature stage and may show chromatoid bodies with pointed or "splintered" ends.

Endolimax nana, present in about 25 per cent of the United States population, is smaller, 6 to 15 μ in diameter in the ameboid stage. The stained nucleus shows no peripheral chromatin but a very large, nearly central karyosome. Movement is sluggishly progressive and bacteria are ingested. The spherical or ovoid cyst is 5 to 14 μ in diameter, containing one to four minute nuclei and sometimes small spherical or rod-like chromatoid bodies.

Iodamoeba williamsi (*bütschlii*), present in 10 to 15 per cent of the United States population, measures 8 to 20 μ in diameter in the ameboid stage. The stained nucleus shows a large central karyosome surrounded by a layer of granules. Movement and inclusions are like those of *E. coli*. The cyst is irregular in shape, 5 to 20 μ in diameter, and contains one or rarely two nuclei. Minute granules may be seen, but the most striking feature of the cyst is a large glycogen mass, staining dark brown with iodine.

Dientamoeba fragilis, a rare parasite in the general population though sometimes common in institutions, is a very small form, 5 to 12 μ in diameter. It usually shows two nuclei, each containing a large multiple karyosome. It moves actively, ingesting bacteria. No cyst stage is known, and the ameboid

stage is apparently responsible for transfer. Dobell[18] has suggested, principally on the basis of its nuclear structure, that *D. fragilis* is a degenerate type of flagellate rather than a true ameba.

Endamoeba gingivalis, probably the first parasitic ameba seen, was reported by Gros[19] in 1849 from the tartar between the teeth. It has no known cyst stage, and is apparently transmitted in the ameboid stage by contact. Formerly suspected of an etiological role in pyorrhea, it is now considered harmless.

CILIOPHORA

Balantidium coli is a large parasite of the intestine of man, monkeys and the pig, usually 50 to 80 μ long by 40 to 60 μ in breadth (Fig. 204). Human infection is widespread but rare. Most of the known human cases show diarrhea
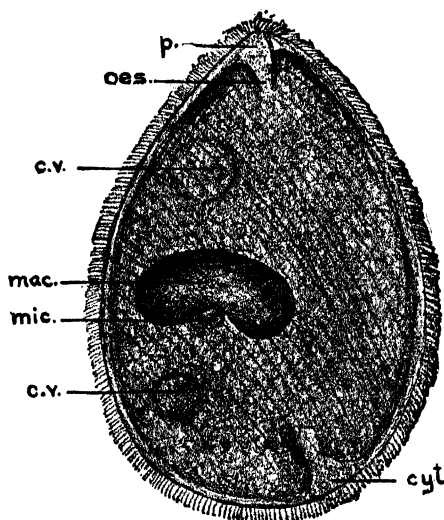
Fig. 204. *Balantidium coli* from man. c.v., Contractile vacuole; cyt., cytopyge; mac., macronucleus; mic., micronucleus; oes., esophagus; p., peristome (Hegner in Hegner and Taliaferro's *Human Protozoology*, courtesy of The Macmillan Company).

or dysentery pathologically like that produced by *E. histolytica*, but carrier infections have been reported.[20] The pig is probably the usual source of human infection, passing resistant cysts which are infective when ingested with contaminated food or water. A variety of laboratory animals, including cats and monkeys, are susceptible, and cultivation has been accomplished in media similar to those used for *E. histolytica*. Carbarsone is said to eliminate infection.[20]

INTESTINAL FLAGELLATES (MASTIGOPHORA)

A variety of species of Mastigophora occurs in the human intestine (Fig. 205). The common species are *Chilomastix mesnili*, *Trichomonas hominis* and *Giardia lamblia*. Only the last is of medical importance. It is occasionally

[18] Dobell: Parasitology, 1940, 32:417.
[19] Gros: Bull. Soc. Imp. Nat., Moscow, 1849, 22 (1 part): 549.
[20] Young: Jour. Amer. Med. Assn., 1939, *113*:580.

associated with diarrhea, usually in children. The best evidence for its pathogenicity in these cases is the fact that quinacrine (atabrine), which eliminates the organism, often cures the intestinal condition.

Trichomonas tenax, often referred to as *T. buccalis*, is a common non-pathogen of the mouth. *T. vaginalis*, occurring in the human vagina, and rarely in the male urethra, is often associated with vaginitis. The evidence for its patho-
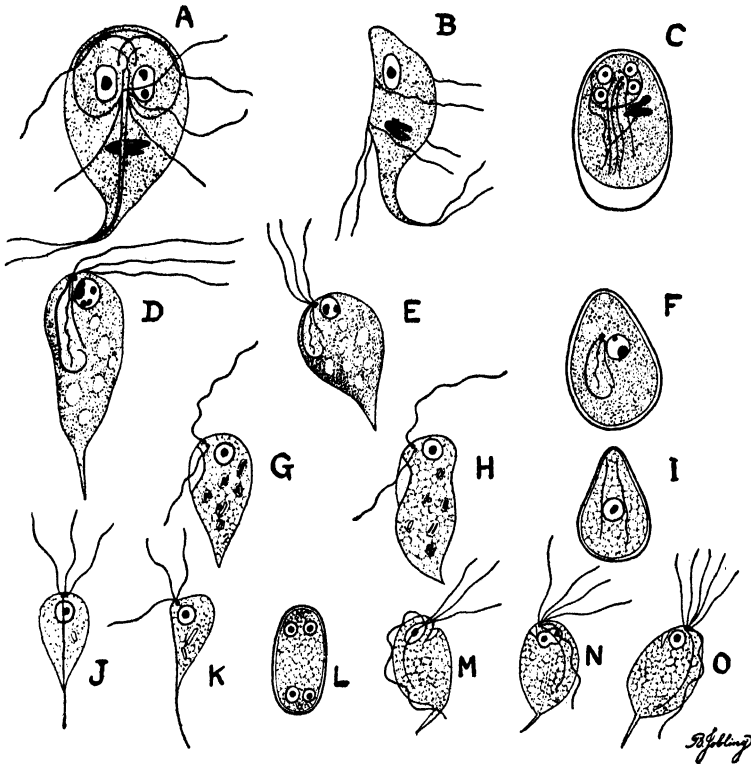
Fig. 205. The flagellates of the human intestine; × 2000. *A–C, Giardia lamblia*, free and encysted forms. *D–F, Chilomastix mesnili*, free and encysted forms. *G–I, Embadomonas intestinalis*, free and encysted forms. *J–L, Tricercomonas intestinalis*, free and encysted forms. *M–O, Trichomonas hominis*, forms with three, four, and five flagella (Wenyon, Proceedings of Royal Institution of Great Britain, March 3, 1922).

genicity is suggestive but inconclusive. The related *T. foetus* is an important cause of contagious abortion in cattle.

Chilomastix and Giardia are spread by cysts from human feces. No cysts are known for Trichomonas, and the relatively resistant flagellated stages are apparently responsible for dissemination. Trichomonas species and Chilomastix grow on various media, but Giardia has not been successfully cultivated.

HEMOFLAGELLATES (MASTIGOPHORA)

The family Trypanosomidae, commonly called hemoflagellates because it includes the mastigophora of the blood and other tissues, comprises several

genera which are parasites of vertebrates, invertebrates or plants. They are classi-
fied on the basis of the morphological types each exhibits. Figure 206 illustrates
these morphological types, of which C may be taken as an example, since it is
the simplest. This form is an elongated blade-like cell body, the cytoplasm stain-
ing blue with blood stains such as Giemsa. Near the center is a round reddish-
violet-staining nucleus. Arising from a small, dark purple-staining granule,
the blepharoplast, at the anterior end is a long whip-like organelle of locomo-
tion, the flagellum. Near the blepharoplast is a larger body, staining similarly,
the parabasal body. This stage of the parasite is known as the *simple flagellate.**
In some forms the simple flagellate stage may round up and lose its flagellum
to become the *nonflagellate* stage shown at D. On the other hand it may meta-
morphose into the *intermediate stage* (B) by posterior migration of the bleph-
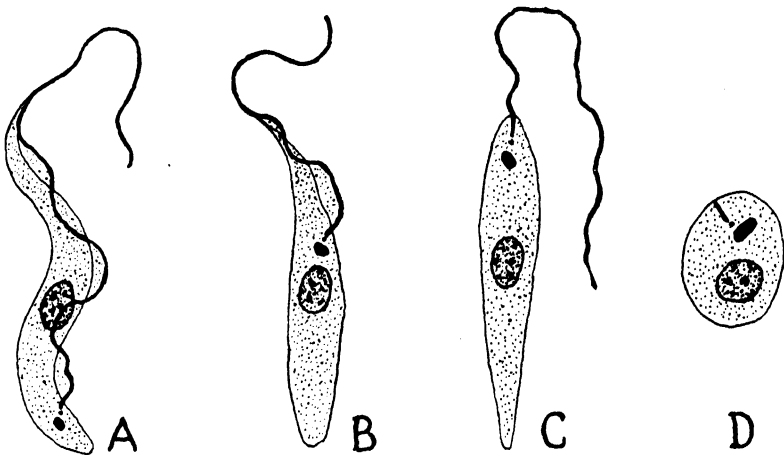
Fig. 206. Morphological types of hemoflagellates. *A*, trypaniform stage. *B*, Intermediate
flagellate. *C*, Simple flagellate. *D*, Non-flagellate stage.

aroplast to near the nucleus. In this stage the flagellar filament is connected
to the body proper by a thin film of cytoplasm, the undulating membrane,
which waves with the flagellar movement, thus acting as a fin. The *trypaniform*
stage (*A*) is attained by further migration of the blepharoplast to near the
posterior end with production of an undulating membrane extending the
whole length of the cell.

The medically important groups of hemoflagellates fall into two genera.
Trypanosoma gambiense and related forms exhibit the trypaniform stage in the
vertebrate host and trypaniform and intermediate stages in the invertebrate.
Trypanosoma cruzi exhibits all four stages in the vertebrate host, trypaniform
and intermediate stages in the invertebrate. Members of the genus Leishmania
exhibit the non-flagellate stage in the vertebrate and the simple flagellate in
the invertebrate host. Reproduction of all forms is by binary fission. The nu-
cleus, blepharoplast and parabasal body divide, a new flagellum arises from one

* Simplified terms are here substituted for the confusing names in general use. Most
texts designate type A *trypanosome*, type B *crithidia*, type C *leptomonas* or *herpetomonas*,
and type D *leishmania*.

blepharoplast, and the cytoplasm divides longitudinally to produce two daughter cells. In the species with which we are concerned, the non-flagellate stages are intracellular in the vertebrate host. The flagellated stages inhabit body fluids of vertebrates or the alimentary tract of insects.

THE TRYPANOSOMES

Trypanosoma Gambiense. Ford[21] observed, in the blood of a Gambian native, a flagellate which was described by Dutton[22] in 1902 as *T. gambiense.* In 1903, Castellani[23] observed similar flagellates in the cerebrospinal fluid of a sleeping sickness patient in Uganda. Bruce and Nabarro,[24] in 1903, transmitted *T. gambiense* to monkeys with a tsetse fly, and Kleine,[25] in 1909, showed that the parasites underwent a cyclic development in the fly.

Characteristics and Life-Cycle. In the blood and lymph of early cases of African sleeping sickness *T. gambiense* exhibits the trypaniform stage, which
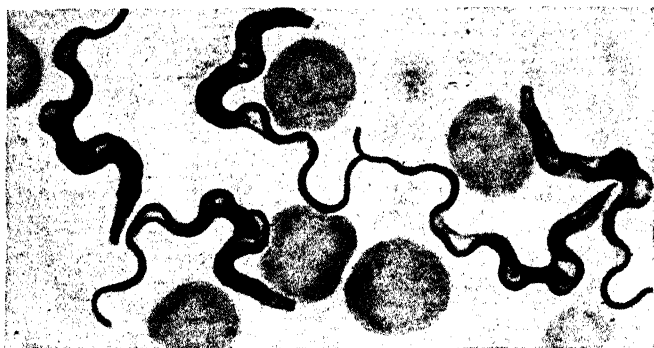
Fig. 207. *Trypanosoma gambiense* in stained blood film; × about 2000. (Kraal.)

in fresh preparations may be seen wriggling among the erythrocytes (Fig. 207). It measures 15 to 40 μ in length, varying from short, broad forms with no free flagellum beyond the undulating membrane to long thin forms with a free flagellum. Reproduction, as in all members of the family, is by binary fission. The transmitting insect, a tsetse fly, becomes infected by ingestion of the parasites in infected blood. In the stomach of the fly those flagellates which survive multiply as trypaniform and intermediate stages, first in the crop, stomach and intestine and later in the salivary glands. Here they form infective parasites, which are injected by the bite of the fly twenty days or more after its infecting blood meal.

On NNN medium,[26] a concentrated blood agar, the parasites multiply to a limited extent. Continuous cultivation has been achieved in some instances, but it is too difficult to be of practical value. Abundant multiplication occurs on the chorio-allantoic membrane of the developing chick embryo.

[21] Forde: Jour. Trop. Med. & Hyg., 1902, 5:261.
[22] Dutton: Thompson-Yate's Lab. Reports, 1902, 4:455.
[23] Castellani: Brit. Med. Jour., 1903, p. 1218.
[24] Bruce and Nabarro: Rep. Sleeping Sickness Comm. Roy. Soc., 1903, 1:11.
[25] Kleine: Deut. med. Wchnschr., 1909, 35:469, 924.
[26] Cf. Craig: *Laboratory Diagnosis of Protozoan Diseases*, Phila. 1948.

African Sleeping Sickness. The incubation period following an infective tsetse fly bite varies from two or three weeks to several months. The first stage of the human disease is a systemic infection in which the parasites are found chiefly in the blood but also in the lymph. Irregular fever is generally the first symptom. Somewhat later, as the parasites come to predominate in the lymph nodes, these organs and the spleen are enlarged, and anemia and wasting are seen. Cardiac injury may be prominent, and edema is often present. This stage gradually gives way to the sleeping sickness stage, in which the parasites are most abundant in the cerebrospinal fluid, occurring less commonly in the lymph. The central nervous system exhibits the prominent lesions, typically resulting in somnolence, apathy and weakness, less commonly in mania and other violent manifestations. Coma and death are the final outcome. Typically the course is of several months' duration, but in parts of East Africa the disease develops more rapidly, invasion of the central nervous system occurs early, and death frequently results from cardiac injury before the typical sleeping sickness stage is reached. In the blood of laboratory animals infected from cases in this area about 5 per cent of the flagellates are short, showing posterior nuclei; this is true only rarely in the parasites from other areas. Because of these differences in morphology and pathogenicity and the fact that transmission involves different species of tsetse flies, Stephens and Fantham[27] separated the East African parasites as a different species, *T. rhodesiense.* Most authors now consider this a local race of *T. gambiense.*

IMMUNITY. Spontaneous recovery is said to occur occasionally in African sleeping sickness. Except for this there are no data available on natural or acquired immunity in man. In rats and mice *T. gambiense* and related parasites produce a rapidly fatal parasitemia, while in guinea pigs and some other hosts they show a characteristic relapsing type of infection. After a period of unchecked multiplication, the organisms are rapidly destroyed, only to reappear in a few days and repeat the cycle. An antibody arising in the serum of a guinea pig at the time of crisis destroys parasites of the strain present before the crisis, while the strain present after flagellates have reappeared is resistant to this antibody. This phenomenon has been observed in animals infected originally with a single parasite, indicating that the change in antigenic structure cannot depend on selection alone but must involve adaptive modification of the parasite.[28]

DIAGNOSIS. Various serum reactions are observable in African trypanosomiasis but are not dependable for diagnosis, chiefly because of the antigenic lability of the parasites. Laboratory diagnosis depends on the finding and morphological identification of the organisms in body fluids, blood, lymph node juice, or cerebrospinal fluid. Dry smears stained with blood stains, such as Wright's, are used, either of the whole fluid as obtained or of centrifuged specimens. In the case of blood, thick or thin films (see p. 779) may be used. The parasites are usually scarce, and a negative finding in any one of the body fluids is of little significance.

Epidemiology and Control. Rare instances of probable coital infection have been reported. However, the normal transfer of infection occurs by the bite of

[27] Stephens and Fantham: Proc. Roy. Soc. London, 1910 (B), 83:28, 85:223.
[28] *Cf.* Fulton and Lourie: Ann. Trop. Med. & Par., 1946, 40:1.

tsetse flies as described above (p. 765). Two species of flies are of most importance, *Glossina palpalis* in West Africa and *G. morsitans* in East Africa, where the more virulent Rhodesian form of the disease occurs. Other species of importance are *G. tachinoides, G. swynnertoni* and *G. pallidipes*. These insects are relatives of the common house fly. They are limited to tropical Africa and a small area in South Arabia, and the human disease is confined to their range. They resemble the house fly in appearance except for the long, narrow proboscis, which is held straight forward from the head, and the manner of folding the wings at rest, flat on the back with one directly above the other. Both males and females bite and can transmit the disease. They bite exclusively by day. The larvae develop completely in the body of the female, are deposited singly on loose soil or sand in well-shaded places, and quickly burrow into the soil to pupate. After four to eight weeks the adults emerge. *G. palpalis* breeds almost entirely near water and is thus more limited in local distribution than *G. morsitans*, which is less dependent on shade and moisture.

The significance of wild animals in spread of African sleeping sickness has long been disputed. Various large wild animals, especially the sitatunga antelope, harbor the flagellates, usually without symptoms, and they have been shown to maintain the infection for long periods in the absence of human reservoirs. However, most authorities agree that man is usually the source of infection.[29] The incidence of human infection varies widely. It is not commonly higher than 2 per cent at present, though in the past villages were observed with infection rates as high as 50 per cent, and catastrophic epidemics have occurred.

The variety of control methods in use testifies to their relative ineffectiveness. Several valuable drugs are available for treatment of the human infection, a method whose success is proportional to the local significance of man as a reservoir of infection. Tryparsamide and other arsenicals are effective in all stages of the disease, though less so in the Rhodesian form, where Bayer 205 (Germanin, Fourneau 309) is more active. This drug is useless in the sleeping sickness stage of the disease but is effective during the first stage and has the particularly valuable property of protecting against infection for at least three months after administration. A new series of compounds represented by stilbamidine and pentamidine shows genuine promise. Pentamidine is said to protect against infection for at least six months.[30] Extensive control programs are under way with mass treatment of diagnosed human cases and their value seems well demonstrated. In some areas mass destruction of the game reservoir has been attempted, but the success of this measure is doubtful. Where the human and animal infection rates are particularly high, wholesale removal of human populations has been carried out. Control of migrants, a perpetual problem in much of Africa, is utilized in some districts in an effort to minimize spread of the disease.

The life-cycle and complex behavior of the tsetse flies make their control exceedingly difficult. Traps and hand-catching have greatly reduced the fly population in some regions. Inspection and fumigation of vehicles have been used to limit the spread of tsetse. The best single method is clearing of forest

[29] *Cf.* Fairbairn: Trop. Dis. Bull., 1948, 45:1.
[30] Van Hoof *et al.*: Trans. Roy. Soc. Trop. Med. & Hyg., 1946, 39:327.

and brush, particularly along streams and around villages, which destroys the
breeding and resting places of the flies.

Trypanosoma Cruzi. Chagas[31] discovered intermediate stage flagellates
in the hind gut of the bug *Triatoma megista* in Brazil. He showed the infec-
tivity of these flagellates for mammals and later found trypaniform stages in
children with a characteristic disease, now known as Chagas' disease. He de-
scribed the organism as *Schizotrypanum cruzi*, having misinterpreted the re-
production in man and animals as schizogony. It is now called *Trypanosoma
cruzi* by most authors.

Characteristics and Life-Cycle. The parasites as seen in the blood of early
cases of Chagas' disease are of the trypaniform type, fundamentally similar to
those of *T. gambiense* but smaller, about 20 μ in length, and with the posterior
end pointed. No multiplication occurs in the blood. The predominant phase
in man and experimental animals is a small, rounded nonflagellate stage, 3 to
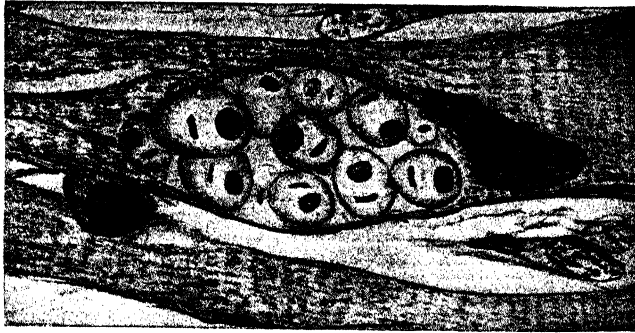
Fig. 208. Non-flagellate form of *Trypanosoma cruzi* in human heart muscle; × 3700
(Taliaferro in Hegner and Taliaferro's *Human Protozoology*, courtesy of The Macmillan
Company).

5 μ in diameter, occurring in clumps in various tissue cells—heart muscle,
macrophages and endothelium (Fig. 208). These organisms have a nucleus
and parabasal body as described above (p. 763). They multiply in the infected
cells by binary fission, producing the dense clumps of organisms mistaken by
Chagas for stages of schizogony. Small numbers of trypaniform stages are ap-
parently continually produced and shed into the circulating blood from the
nests of intracellular non-flagellate parasites.

Various bugs of the family Reduviidae acquire the infection from a blood
meal containing the trypaniform stages. These become intermediate flagellates
in the mid- and hindgut, where they multiply to produce infective trypaniform
flagellates two to three weeks after the infecting blood meal. These organisms
are shed in the feces of the bug. They infect man by entering the wound
caused by the bite of the infected bug or by penetrating the mucosae, par-
ticularly of the mouth or eye.

Continuous culture of *T. cruzi* on NNN medium and in simpler broth
media is relatively easy. The parasite multiplies in the water of condensation as
trypaniform and, predominantly, intermediate flagellates. The non-flagellate

[31] Chagas: Mem. Inst. Oswaldo Cruz, 1909, *1*:159.

stages have been grown in tissue cultures of macrophages from infected animals.[32]

Chagas' Disease. Chagas described the infection from a region having serious endemic goiter, and most of his cases had marked thyroid pathology. It has since been shown in other regions that the goitrous manifestations he described are not part of Chagas' disease. The incubation period is one to two weeks. Irregular fever and edema, particularly of the eyelids (Romaña's sign) characterize the acute phase, and there is considerable enlargement of lymph nodes, spleen and liver toward the end of this period. The acute disease is rare except in small children, in whom occur almost the only deaths attributable to the infection. The chronic disease, which occurs in adults or following the acute stage in children, varies in symptomatology with the localization of the parasites but is most often characterized by myocarditis caused by heart muscle infection.

IMMUNITY. Evidence concerning acquired immunity is lacking in the human disease, but experimental animals are immune to reinfection after recovery. Serum from such animals partially protects against experimental infection. The serum of human cases fixes complement in the presence of extracts of infected tissues or cultures of *T. cruzi*, and the complement-fixation test, the Machado reaction and various modifications, is widely used for laboratory diagnosis.

DIAGNOSIS. Laboratory methods for diagnosis other than the complement-fixation reaction depend on the demonstration of the parasites. During the early stages of the human disease trypaniform flagellates may be found in stained blood smears. Later, indirect evidence of the presence of parasites may be obtained by blood culture or by "xenodiagnosis" (host diagnosis), in which laboratory-reared bugs become infected after feeding on a case.

Epidemiology and Control. Natural infection is normally acquired from infected bugs as described above, although occasionally the disease is acquired by direct contamination of mucosae, as in the congenital infection of infants. The chief vectors are *Triatoma (Mestor) megista* and *Rhodnius prolixus*, but about forty species of the family Reduviidae have been incriminated. These insects are members of the order Hemiptera, or True Bugs, to which belong also bed bugs, chinch bugs and many others. They are large insects with an elongated, cone-shaped head. Most species are predatory on other insects but some live on vertebrate blood. They inhabit the nests of various animals and may occur in human houses of poor construction, where males and females commonly bite sleeping persons about the mouth or eyes. The total life-cycle, involving egg, larval, nymphal and adult stages, occupies six to ten months.

The human disease is widespread in South and Central America but of low incidence in most areas. Various mammals are susceptible and the infection occurs naturally in many of these, particularly dogs, cats, wild rodents, opossums and armadillos, all of which serve as reservoirs of human infection. In the southwestern United States natural infections occur in the insects and in the wild rodents which they infest, but human infections have not been reported in this country.

Successful control of Chagas' disease has not been attained. The most prom-

[32] Hawking: Trans. Roy. Soc. Trop. Med. & Hyg., 1946, 40:345.

ising methods involve destruction of infected domestic animals and improvement of human houses to exclude the insects and the reservoir hosts. There is no successful chemotherapy for Chagas' disease, the drugs useful in African trypanosomiasis being inactive in this infection.

Roskin, Klueva and others have reported that extracts of *T. cruzi* cause regression of malignant tumors. Other workers have verified the beneficial effect of *T. cruzi* infection in such tumors but have not consistently observed activity of extracts of the parasites.[33]

Other Species of Trypanosoma. A number of important diseases of domestic animals are caused by species of Trypanosoma similar to *T. gambiense*. Nagana is a rapidly fatal disease of the horse family, and to a less extent of cattle and dogs, occurring in a wide area of East Africa. It is caused by *T. brucei*, an organism very similar to the Rhodesian form of *T. gambiense*, and transmitted mostly by *G. morsitans*. Wild game are commonly infected without detectable symptoms and are an important reservoir. A number of other species transmitted by tsetse flies are important disease agents of animals in Africa. *T. evansi* causes a disease of horses, camels and mules known as *surra*, which is widespread in Asia, extending to Russia, Arabia and Madagascar. It is transmitted mechanically by horse flies and stable flies. Related species cause equine diseases in South America, one of which, *T. hippicum*, is transmitted by vampire bats. *T. equiperdum* causes a disease of the horse family known as *dourine*, of nearly world-wide distribution. The disease is known as "horse syphilis" because of a general similarity in course of infection and the fact that spread occurs by coitus.

Two rodent species, *T. lewisi* of rats and *T. duttoni* of mice, are of especial interest. They are found in various parts of the world and are easily obtained for laboratory study. Like *T. gambiense* they occur in the vertebrate host only in the trypaniform stage. They resemble *T. cruzi*, however, in that multiplication in the invertebrate hosts, fleas and sucking lice, takes place in the mid- and hindgut, and infection of the vertebrate is acquired from flagellates in the insects' feces. Host specificity is very strict, and although the parasites are readily cultivable on NNN medium they do not multiply appreciably in developing chick embryos as do flagellates of the *T. gambiense* type. In rats inoculated with blood containing *T. lewisi* the flagellates multiply unchecked for several days, showing the great size variation characteristic of rapidly reproducing populations. This variation decreases rapidly with the appearance of a non-absorbable antibody, an *ablastin*, which completely inhibits reproduction. Within a few days a trypanolysin destroys a majority of the parasites, the remainder persisting in the peripheral blood without multiplication until, after one to several months, another trypanolysin eliminates the survivors.[34]

THE LEISHMANIAS

Leishmania Donovani. Leishman[35] and Donovan,[36] in 1903, described oval parasites of the macrophages in cases of kala-azar in India. These were

[33] *Cf.* Hauschka *et al.*: Jour. Nat. Cancer Inst., 1947, 7:189.
[34] Taliaferro: Jour. Exp. Med., 1924, 39:171.
[35] Leishman: Brit. Med. Jour., 1903, p. 1252.
[36] Donovan: Brit. Med. Jour., 1903, p. 1401.

recognized as mastigophora when Rogers[37] showed they developed into motile flagellates in culture.

Characteristics and Life-Cycle. In the human disease, kala-azar, the parasites occur as non-flagellate stages, 3 to 5 μ long, in macrophages, where they resemble the non-flagellate stages of *T. cruzi* (Fig. 209). They multiply by binary fission until the cytoplasm of the host cell is crowded, when they escape to infect new cells. While the parasites predominate in internal organs, they occur in the skin macrophages as well, and it is probably from this site that the intermediate hosts, sandflies of the genus Phlebotomus, become infected. The parasites transform into the simple flagellate form, 14 to 20 μ long, and multiply in the mid- and foregut of the insect, which becomes infective after
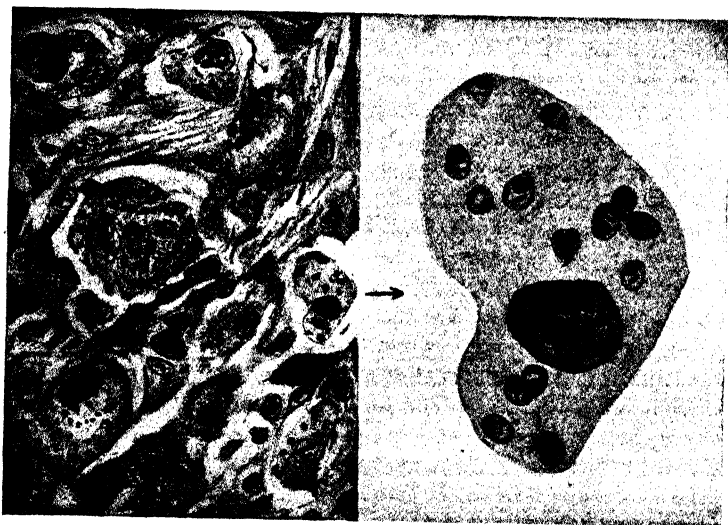
Fig. 209. *Leishmania donovani* in the human spleen. *A*, Section of spleen showing five macrophages infected with the parasites. *B*, One macrophage enlarged to show structure of the parasites. *A*, $\times$ about 550; *B*, $\times$ 2500 (Taliaferro in Hegner and Taliaferro's *Human Protozoology*, courtesy of The Macmillan Company).

a week or more. The simple flagellates, injected by the bite of the fly into a new host, reestablish the vertebrate phase of the cycle.

L. donovani is easily cultivated in NNN and other media at 22° to 35° C., multiplying in the simple flagellate stage as in the insect host (Fig. 210). In tissue cultures of spleen from infected animals the non-flagellate stages multiply abundantly.[38]

Kala-Azar. Visceral leishmaniasis, or kala-azar, is usually a chronic disease. Typically it begins, after an incubation period of one to four months (sometimes much longer), with a high temperature, which merges into an irregular fever showing long swings of temperature. The spleen and liver enlarge greatly with hyperplasia of the parasitized macrophage system. Wasting, emaciation and edema of the legs are common. Dysentery often occurs as a result of heavy

[37] Rogers: Lancet, 1904, p. 215.
[38] Hawking: Trans. Roy. Soc. Trop. Med. & Hyg., 1948, 41:545.

infection in the intestinal wall. The skin is typically dusky in hue, whence the name kala-azar, meaning "black fever." The skin is infected with the parasite but usually does not show lesions until months after systemic recovery when depigmented areas appear, later often becoming slightly raised papules. Anemia and leucopenia are characteristic. Death is the rule in untreated cases, usually as a result of secondary infection.

IMMUNITY. Treated or spontaneously cured kala-azar is apparently followed by a lasting, solid immunity, for second infections are exceedingly rare. However, vaccines have failed to protect against or ameliorate the disease. Complement-fixing and agglutinating antibodies are detectable in active and recovered cases, but they give cross-reactions with cutaneous leishmaniasis.

DIAGNOSIS AND TREATMENT. Crucial laboratory diagnosis of kala-azar may be obtained by the finding of parasites in biopsies of skin, spleen, liver or bone

Fig. 210. *Leishmania donovani;* simple flagellate forms, from a culture; Wright's stain; × 1800 (Bulletin No. 1, Office of the Surgeon General, Washington, January, 1913).

marrow or in scrapings from the nasal mucosa. Sternal bone marrow puncture is a reliable and safe procedure. Blood cultures may be positive. In addition, a group of non-specific serological reactions has shown great value. These reactions depend on the fact that the serum euglobulin is greatly increased in amount in cases of kala-azar. In the formol-gel test (Napier's aldehyde reaction), positive sera form an opaque gel when formalin is added. In the antimony test a pentavalent antimonial drug causes a heavy flocculent precipitate in sera of cases. These tests are positive in over 80 per cent of cases and give false positives only occasionally with other diseases, especially schistosomiasis and malaria.

Several antimony compounds, particularly neostibosan and solustibosan, are effective in kala-azar. Successful treatment is often followed by dermal leishmaniasis, which may persist for several months. The drugs stilbamidine and pentamidine mentioned in connection with African sleeping sickness (p. 766) are also effective in kala-azar.

Epidemiology and Control. Kala-azar occurs in China, India, South Russia,

Mesopotamia, the Mediterranean littoral, Equatorial Africa, Eastern Brazil and Northern Argentina. In Asia it is apparently a disease of man, though various lower animals, particularly dogs, hamsters and mice, are susceptible. Infection may occur at any age but is most common in older children and young adults. In the Mediterranean area, however, it is primarily a disease of small children. Here dogs are commonly infected and apparently serve as the reservoir, since, unlike children, they show abundant parasites in the skin. The Mediterranean parasite is called by some authors L. *infantum* and the South American form has been described as L. *chagasi,* but both are generally believed to belong to the species L. *donovani.*

Factors of seasonal and geographical distribution early pointed to sandflies as probable vectors, and it was soon shown that they were susceptible, developing heavy intestinal infections when fed on cases.[39] However, fifteen years of experimentation with thousands of flies and hundreds of test animals produced only four transfers by bite. Other insects, particularly fleas and bedbugs, were studied and eliminated as vectors. Different means of spread were suggested by the demonstration of parasites in nasal secretions, urine and feces of cases. It was shown that hamsters could be infected by mouth. Direct transmission may occur but is probably uncommon in view of the epidemiology of the disease. Recently, new evidence has made it virtually certain that sandflies are the significant vectors. Smith *et al.* maintained infected sandflies on fruit juices instead of the customary blood meals, and infections have consistently developed in hamsters and mice bitten by such flies.[40] Similar transmission has since been reported with human volunteers.[41] In these flies the pharynx often becomes blocked with the simple flagellate stages, a phenomenon not observed in blood-fed flies, and in their vigorous efforts to feed the "blocked" flies apparently are more likely to inject parasites.

The sandfly vectors belong to the genus Phlebotomus of the family of small flies, Psychodidae. The most important species are P. *argentipes* in India, P. *chinensis* in China, and P. *major* and P. *perniciosus* in the Mediterranean region. The vectors in other areas are as yet unknown. The adult flies are minute, night-biting insects. They fly weakly and travel very short distances but penetrate ordinary window-screening with ease. The larvae develop in loose damp soil or debris, mostly in cracks in walls, cliffs, caves, etc., the entire life-cycle requiring one to two months.

Control of the transmitting insects is difficult, though some success has resulted from cleaning up potential breeding places near human habitation. DDT sprayed on breeding areas has shown promise as a tool for the reduction of sandflies. Fine-mesh bed nets, fans and repellents are of value as protection against the bites of the flies. Large-scale treatment of human cases is widely used for control.

Leishmania Tropica. L. *tropica* was discovered by Wright,[42] in 1903, in an Armenian patient in Boston. Morphologically and culturally identical

    [39] Knowles, Napier and Smith: Ind. Med. Gaz., 1924, 59:593.
    [40] Smith, Halder and Ahmed: Ind. Jour. Med. Research, 1940, 28:575; *ibid.,* 1941, 29:783, 799.
    [41] Swaminath, Shortt and Anderson: Ind. Jour. Med. Res., 1942, 30:473.
    [42] Wright: Jour. Med. Res., 1903, 10:472.

with *L. donovani*, it differs in infecting primarily the skin, where it proliferates in the macrophages of the subcutaneous tissue. In man, dogs and wild rodents it produces large single or multiple ulcers, usually of exposed parts of the skin, known as oriental sores. These lesions appear after an incubation period of ten days to several months. They increase to a diameter of 1 to 3 cm. and heal spontaneously after several months to leave a disfiguring scar. Permanent immunity follows infection, and deliberate induction of sores on unexposed parts of the body has long been practiced in the Middle East to avoid the disfigurement of exposed scars. Transfer by direct contact, fomites and house flies is possible, but the principal method of transmission is undoubtedly by sandflies in a manner similar to kala-azar. Oriental sore occurs in Southern Asia, Southern Russia, the Near East, Equatorial Africa and the Mediterranean region, the principal vectors being *P. sergenti* and *P. papatasii*. Laboratory diagnosis is made by identification of the parasites in stained smears of scrapings from the lesions. Reliable diagnosis of oriental sore has recently been reported with an intradermal test.[43] Since it is often very difficult to detect parasites in the lesions, such a procedure would be valuable. Local or systemic administration of antimony compounds such as neostibosan is usually effective in treatment.

Leishmania Braziliensis. *L. braziliensis* resembles *L. tropica* but often shows a predilection for mucocutaneous borders, as around the mouth and nose. Here it produces an eroding lesion, which lasts for months. Extension to the pharynx may result in death. The disease, known by various names, of which uta and espundia are the most common, has a wide distribution in South America, extending to Southern Mexico.

<div align="center">SPOROZOA</div>

Malarial Parasites. With the exception of a rare intestinal parasite, the only sporozoa infecting man are the malarial parasites. They were first recognized by Laveran[44] in 1880, and the life-cycle in human erythrocytes was described by Golgi.[45] Manson's suggestions led Ross[46] to the demonstration in 1898 of mosquito transmission of avian malaria, and Grassi, Bignami and Bastianelli[47] later in the same year showed the mechanism of spread of human malaria. Man harbors three or possibly four species of malarial parasites, of which *Plasmodium vivax* will serve as an example.[48]

Characteristics and Life-Cycle. In fresh preparations of infected blood the parasites of *P. vivax* appear as clear areas in the erythrocytes. They contain yellow or brown granules of pigment, a digestion product of hemoglobin which has been identified microchemically as hematin. In direct blood films colored with stains such as Giemsa the parasites show blue cytoplasm and violet red nuclei (Fig. 211). The earliest stage in the erythrocyte, the *ring* stage, consists of a thin ring of cytoplasm with a nucleus at one side. The para-

[43] Dostrovsky and Sagher: Ann. Trop. Med. & Par., 1946, 40:265.
[44] Laveran: Bull. Acad. Med., 1880, 9:1235, 1268, 1346.
[45] Golgi: Arch. Sci. Med., 1889, 13:173.
[46] Ross: Ind. Med. Gaz., 1898, 33:401, 448.
[47] Grassi, Bignami and Bastianelli: Atti. R. Accad. Lincei Rendic. Ser. 5, 1898, 8:21; *ibid.*, 1899, 8:100, 434.
[48] An excellent comprehensive book is: Russell, West and Manwell: *Practical Malariology*. W. B. Saunders Co., Philadelphia. 1946.
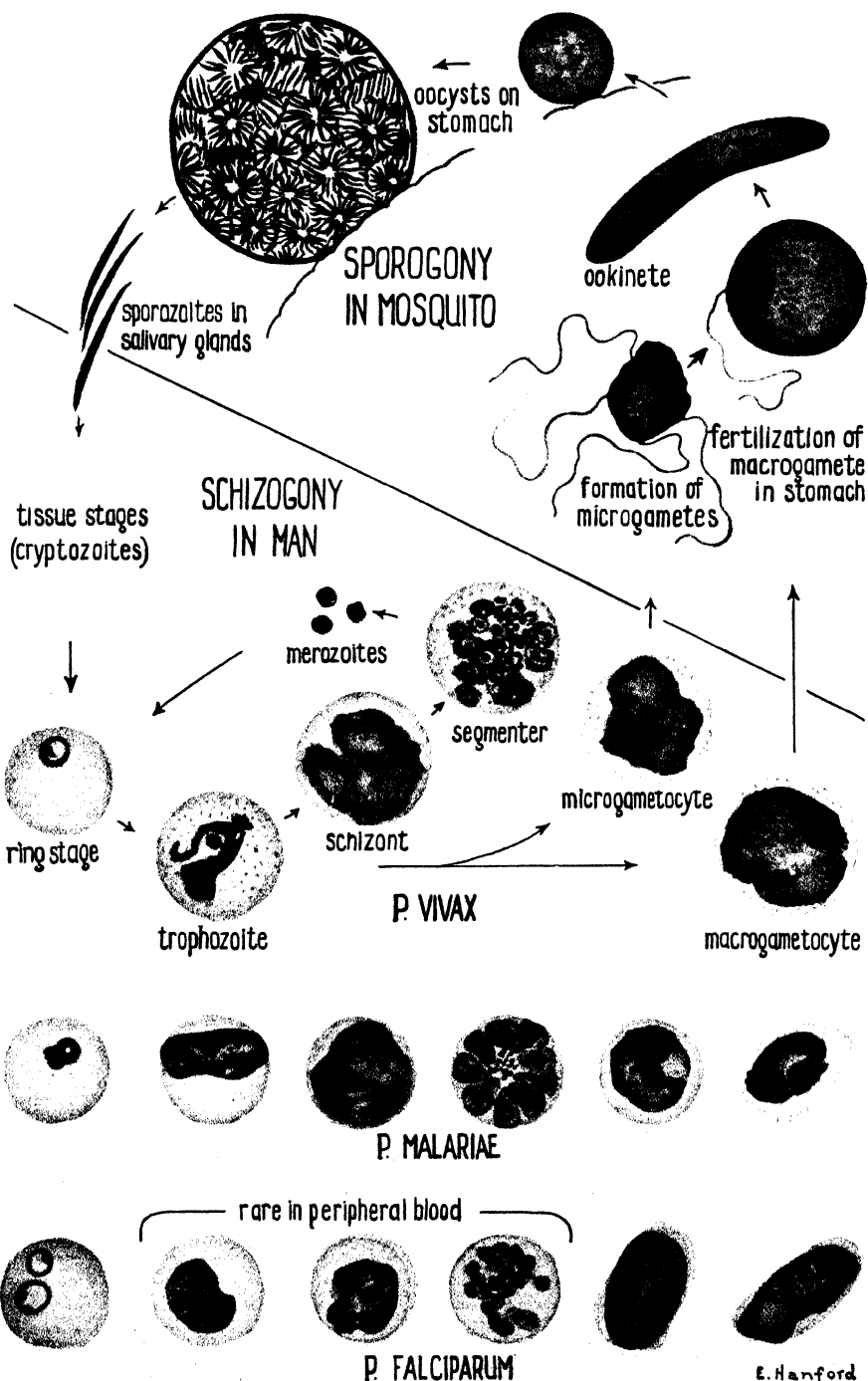
Fig. 211. Life-cycle and comparative morphology of the malarial parasites of man. (Schizogonic stages redrawn from Huff, *Manual of Medical Parasitology*). Oocysts × 600; remaining stages × 2000.

site grows, becoming an irregular uninucleate body containing several brown pigment granules. This stage is known as the *ameboid trophozoite*. The parasitized cell has now enlarged somewhat and may show scattered throughout its cytoplasm minute red granules, "Schüffner's dots," which are apparently a result of injury to the cell. The parasite continues to grow, accumulating more pigment. Eventually it nearly fills the erythrocyte, which is now about one and one half times its normal diameter. The nucleus divides repeatedly until twelve to twenty-four, usually about sixteen, nuclei are present. This is the *schizont* stage. Finally, in the *segmenter* stage, the cytoplasm divides, a portion surrounding each nucleus. The pigment is left in a dense clump, and the cell disintegrates to release the daughter cells, or *merozoites*, into the plasma. Here they invade fresh erythrocytes and repeat the cycle. The above process of growth and multiple fission is known as schizogony. It occupies, in *P. vivax*, about forty-eight hours, and the growth is regulated by the daily cycle of activity of the host, so that segmentation usually occurs at about the same time every other day.

After several schizogonic cycles a difference may be noted in the infection. Some ameboid stages, instead of becoming schizonts and undergoing asexual reproduction, develop into large uninucleate parasites with scattered pigment granules. These are the sexual stages. The female, or *macrogametocyte,* shows a compact, dark red nucleus and intense blue cytoplasm. The male, or *microgametocyte,* has a more diffuse, less deeply-stained nucleus and the cytoplasm is paler, often pinkish rather than blue. The gametocytes undergo no further development in man, eventually degenerating or being destroyed unless they are taken up by a susceptible mosquito.

In the stomach of the mosquito, gametes are produced. The macrogametocyte escapes from the erythrocyte and becomes a single *macrogamete,* corresponding to a metazoan ovum. The microgametocyte produces at its surface, by a process generally called "exflagellation," four to eight long, whip-like *microgametes,* counterparts of the spermatozoa of higher animals. One of these actively wriggling microgametes fertilizes a macrogamete. The resulting zygote elongates, becoming a motile *ookinete* about 20 μ long. The above process in the mosquito occupies one to two days. Many ookinetes are destroyed in the mosquito stomach, but some penetrate its wall and come to rest as round *oocysts* on the outside of the stomach. Here they grow, undergoing nuclear multiplication, until a diameter of about 50 μ is attained, the oocyst now containing many hundreds of nuclei. Each acquires a bit of cytoplasm and becomes a spindle-shaped body, about 8 μ long, the *sporozoite.* With the rupture of the oocyst, these sporozoites scatter throughout the body of the mosquito. Many accumulate in the salivary glands, where they are injected into man by the biting mosquito. The complete development in the mosquito requires from one to two weeks. 25° C. is said to be the optimal temperature, no development occurring below 15° C. nor above 30° C.

It is only recently that direct evidence has been presented on the early development of *P. vivax* in man. Schaudinn,[49] in 1902, described penetration of the sporozoites into erythrocytes and initiation of schizogony. Later evidence cast serious doubt on his observations. First, many investigators failed

[49] Schaudinn: Arb. a. d. Gesundheitsamte, 1902, *19*:169.

to confirm Schaudinn's observation. Second, it was not possible to infect normal individuals by transfusion of blood drawn from infected persons during the first part of the incubation period. Third, drugs highly effective against the parasites in erythrocytes had no influence on the infection when given during the early incubation period. These facts suggested that the parasites present during the incubation period were unlike those seen later in erythrocytes and were not present in the peripheral blood.[50]

In several species of avian malaria the steps from inoculation of sporozoites to infection of the erythrocytes have been clearly described.[51] In these infections sporozoites inoculated into the skin enter subcutaneous macrophages. Here, as *cryptozoites*, they undergo a type of schizogony fundamentally like that in erythrocytes except that no pigment is produced. In twenty-four to
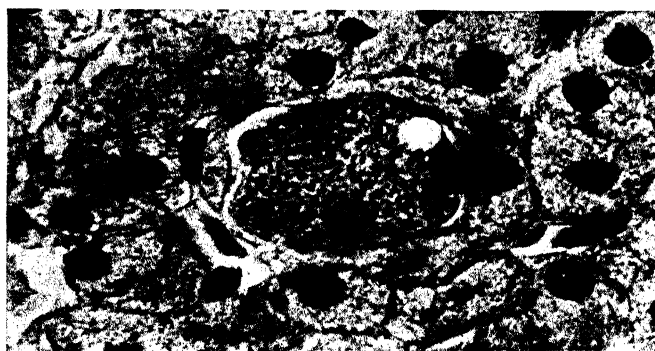
Fig. 212. Cryptozoite of *P. vivax* in liver section. (Courtesy of Col. H. E. Shortt.)

forty hours segmentation occurs, producing merozoites which invade new macrophages and repeat the cycle. Some of the second generation merozoites enter erythrocytes to establish the blood schizogony. Others continue to reproduce in tissue cells as *exoerythrocytic* stages. These exoerythrocytic stages apparently continue to reproduce during the whole course of infection and may repeatedly give rise to new schizonts in erythrocytes. The cryptozoites and later erythrocytic stages are resistant to drugs effective against the blood parasites. Their existence, therefore, offers an explanation of the phenomena discussed in the preceding paragraph.

Until recently neither cryptozoites nor later exoerythrocytic stages had been observed in mammalian malarias. Shortt *et al.*[52] have produced indisputable evidence for the existence of such parasites in a monkey malaria and, later, in *P. vivax*. Large schizonts (Fig. 212) were found in the liver five to ten days after sporozoite inoculation. Unlike those in avian malarias, the cryptozoites in *P. vivax* were said to be in hepatic cells. The parasites observed appear to constitute a second generation of reproduction, but no organisms have been observed before the fifth day of infection. In one monkey schizonts have been observed in the liver three and one-half months after infection, indicating persistence of the tissue parasites during and after the acute blood infection.

[50] *Cf.* Fairley: Trans. Roy. Soc. Trop. Med. & Hyg., 1947, 40:621.
[51] Huff and Coulston: Jour. Infect. Dis., 1944, 75:231.
[52] Shortt and Garnham: Trans. Roy. Soc. Trop. Med. & Hyg., 1948, 41:785.

While much study is still required in this very important aspect of malaria, it is now possible to reconstruct in essence the development of infection in *P. vivax*. Sporozoites initiate a type of schizogony similar to that described above for avian malarias. In *P. vivax*, however, at least part of this cycle seems to be in liver cells rather than macrophages. After about nine days some of the merozoites initiate the erythrocytic schizogony. Others apparently continue the exoerythrocytic infection, which can later give rise to new generations of erythrocytic parasites.

In drawn blood with added dextrose malarial parasites persist for a few days and may multiply slightly.[53] New techniques have permitted cultivation of *P. vivax* and a monkey malaria for several generations in erythrocytes.[54] While the methods are too elaborate for diagnostic use, they have permitted extensive study of the physiology of the parasites.[55] Exoerythrocytic stages of several avian malarias have been successfully grown in tissue cultures of macrophages from infected birds.[56]

The life-cycles and morphology of the other human malarial parasites in erythrocytes are fundamentally similar to those of *P. vivax* (Fig. 211). *P. malariae* requires seventy-two hours for the completion of schizogony. The erythrocytic parasites show several differences of diagnostic value. No granules of the type of Schüffner's dots are seen in the infected cells. The cells are not enlarged during growth of the parasites. The older trophozoite is not ameboid like that of *P. vivax* but commonly exhibits a "band" form across the parasitized cell. The segmenter produces six to fourteen, usually eight, merozoites, arranged in a "rosette" form. The gametocytes are small and the pigment typically occurs in abundant, large dark granules. The optimal temperature for sporogony in the mosquito is said to be about 22° C., and sporogony is slow, requiring three weeks or more.

P. falciparum, like *P. malariae*, causes no enlargement of the infected cells, but some parasitized cells show granules, Maurer's dots, which correspond to the Shüffner's dots of *P. vivax*. Schizogony results in six to twenty-four merozoites. The schizonts and segmenters typically accumulate in capillaries of internal organs, appearing in the peripheral blood only in very heavy infections. Normally, therefore, only ring stages and gametocytes are seen in blood films. Infection of erythrocytes by two or more rings is common and the rings often contain two nuclei. The gametocytes are distinctive, exhibiting a characteristic sausage shape which distorts or obliterates the infected cell. Because of their shape, they are commonly called "crescents." The pigment is finely granular or amorphous in appearance, and in the gametocytes it is typically concentrated about the nucleus. The optimal temperature for sporogony is said to be 29° C.

P. ovale is a rare species showing similarities to both *P. vivax* and *P. malariae* but characterized chiefly by the fact that the infected cell is often distorted into an oval. Its significance is not known, although many investigators consider it an aberrant type of *P. vivax*.

[53] Bass and Johns: Jour. Exp. Med., 1912, *16*:567.
[54] Geiman *et al.*: Jour. Exp. Med., 1946, 84:583.
[55] Anfinsen *et al.*: Jour. Exp. Med., 1946, 84:607.
[56] Hawking: Trans. Roy. Soc. Trop. Med. & Hyg., 1945, *39*:245.

Malaria. Vivax malaria, or *benign tertian malaria,* is featured by typical chills and fever occurring at the time of segmentation of the peripheral blood parasites. These paroxysms begin with an acute, shaking chill while the temperature is rising. A "hot stage" occurs at the fever peak, the patient feeling unbearably hot and the oral temperature usually reaching 104° or 105° F. This gives way to a "sweating stage" during which the fever falls rapidly to normal or slightly below. Parasite products or red blood cell contents released at disruption of the infected cells are presumably responsible for the paroxysm. Paroxysms coincide, as stated above, with the time of parasite segmentation. Hence they occur every forty-eight hours if all the parasites reproduce on the same day. Often, however, distinct broods of parasites segment on alternate days. Vivax malaria commonly shows quotidian (daily) chills for several days followed by the suppression of one brood of parasites with resultant tertian chills (every other day). Between paroxysms the patient feels and appears normal.

Significant anemia occurs commonly in vivax malaria but is rarely serious unless other factors are involved. A week or more after the beginning of an attack, particularly in children, the spleen usually enlarges and remains enlarged for two to six months after termination of the attack. The untreated attack typically lasts three to six weeks, often with temporary cessations of clinical activity. It is followed by a period of latency, which probably lasts two to three years as a rule, during which parasites cannot be found microscopically in the peripheral blood. During part of this time large transfusions of blood from the infected person fail to induce infection in recipients.[57] This suggests that exoerythrocytic stages are responsible for maintenance of infection during latency. In about one-third of cases renewal of clinical activity, or relapse, occurs during this period, the clinical picture resembling that in the primary attack.

Malaria due to *P. malariae* is known, because of the seventy-two hour cycle of reproduction, as *quartan malaria,* the chills occurring every third day. It is basically similar to vivax malaria, though the paroxysms are often more severe. The incubation period is usually long, three weeks or more, and the period of clinical activity typically lasts for several months. Relapses are uncommon, but latent infection may persist for many years, as shown by the occasional development of quartan malaria in recipients of blood transfusions from persons who have not shown evidence of infection for thirty years or more.

Falciparum malaria is widely known as *malignant tertian* or *estivo-autumnal malaria.* As noted above, *P. falciparum* is characterized by accumulation of schizogonic stages in internal organs. As a result, in addition to or instead of the typical paroxysms, which are quotidian or tertian, various local manifestations of the disease may be prominent. This is especially true in the tropics where such "pernicious" malaria may follow a series of typical chills. The most common types are algid (cold) malaria, gastro-intestinal manifestations, and cerebral malaria with coma and often death. These peculiarities of falciparum malaria make it the cause of most of the deaths attributable to human malarial infection and an important factor in many deaths traced to other diseases. Blackwater fever, a dangerous hemoglobinuria, is associated with falciparum

[57] *Cf.* Fairley, ref. 50.

malaria and is thought to be an occasional late effect of repeated infection. *P. falciparum* infections relapse uncommonly and rarely persist more than a year or two.

IMMUNITY. Malaria exhibits what has been termed "infection immunity." After recovery from an acute attack, the individual is highly resistant to super-infection with a strain similar to that already present in his body, but the resistance lasts for at most a few months after elimination of parasites from the body. This immunity depends on active phagocytosis and digestion of parasitized erythrocytes by the macrophages of spleen, liver and bone marrow.[58] That the parasitized cells are sensitized by antibodies is suggested by the fact that serum from recovered animals partially protects against infection.[59] The immunity to superinfection is species- and strain-specific. The latent malarial infection is a state of balance between reproduction of the parasites and their destruction by the body defenses. Relapse occurs when this balance is disturbed, permitting the parasites to increase greatly in numbers. The factors are little known, though it has recently been shown that operations increasing blood sugar induce relapse in avian malaria.[60]

Different strains of vivax malaria exhibit characteristic patterns of activity. Thus, an American strain typically relapses about ten months after the initial infection, while a strain from New Guinea usually relapses within two months.[61] Data such as these indicate that reactivation of latent malaria is at least partly determined by inherent cyclic properties of the parasites.

Partial inhibition of avian and monkey malarial infections has been achieved with killed vaccines. There is no evidence concerning human malaria, but, because of the nature of immunity to malaria, it seems unlikely that vaccination would be of practical value. Serum agglutination of parasitized cells or isolated parasites has been reported in experimental infections. It is species- or strain-specific. Complement-fixing antibodies occur in the serum and are group-specific, reacting with extracts of avian and simian malarial parasites as well as with those from man.

DIAGNOSIS. Laboratory diagnosis of acute malaria rests on the finding of parasites in peripheral blood films. They are most easily identified in thin films stained with Giemsa, but, since parasites are often scanty in the peripheral blood, dehemoglobinized thick films, similarly stained are widely used. The rapid staining method of Field[62] is of especial value for such preparations. The fact that parasite morphology is abnormal in thick films is offset by the much greater volume of blood which can be examined in a comparable time. In surveys the characteristic spleen enlargement is a non-specific but highly suggestive indication of current or recent malarial infection. Because it usually persists for several months it affords a more stable index of the infection rate in an area than do clinical or parasitological surveys.

CHEMOTHERAPY. Treatment of malaria has for centuries depended on the bark or extracts of the bark of cinchona trees. The best known alkaloid is

[58] *Cf.* Taliaferro: *Human Malaria*, p. 239.
[59] Coggeshall and Kumm: Jour. Exp. Med., 1937, 66:177.
[60] Gajewsky and Tatum: Jour. Infect. Dis., 1944, 74:85.
[61] Coatney *et al.*: Amer. Jour. Hyg., 1948, 47:120.
[62] Field: Trans. Roy. Soc. Trop. Med. & Hyg., 1940, 33:635, 34:195.

quinine, though others are effective against the parasites. Quinine neither prevents nor cures the natural infection but rapidly suppresses the number of blood parasites below the density necessary to produce symptoms. The synthetic drug *quinacrine* (atabrine) has similar action and in addition it can cure falciparum malaria. *Chloroquine*, developed in large-scale World War II research, has properties like those of quinacrine but is more effective.[63]
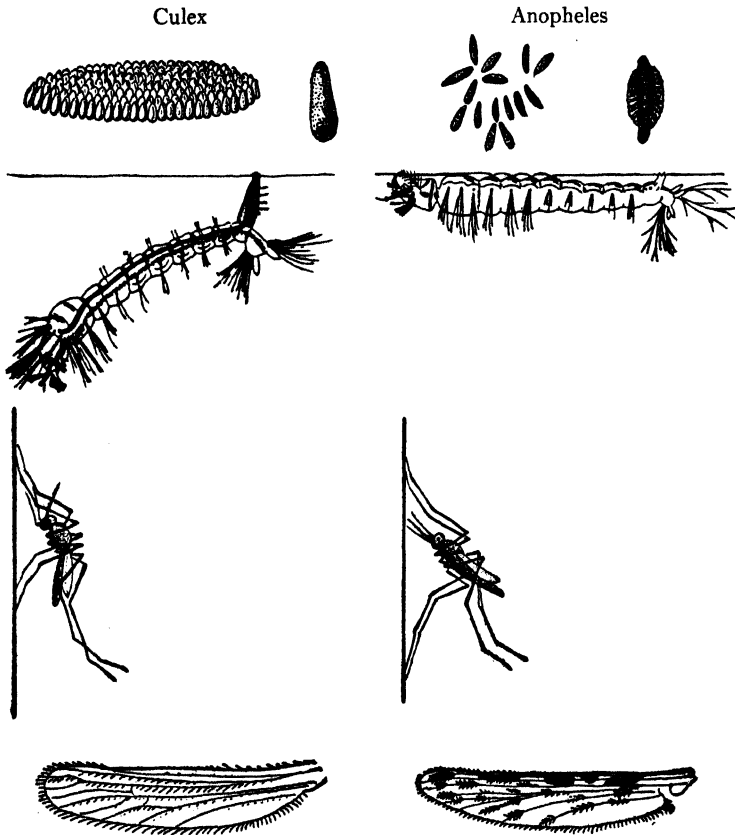
Fig. 213. Comparison of Culex and Anopheles. Eggs, larvae (note position), position of insects at rest, wings (Kolle and Hetsch, partially redrawn).

Paludrine, a product of British wartime research, is a very effective suppressive for vivax malaria and is both prophylactic and curative for *P. falciparum* infection.

The above drugs, especially chloroquine and paludrine, taken continuously during exposure to vivax malaria act as suppressives, holding the infection down to sub-clinical levels. They do not prevent or cure the infection. Recent observations have confirmed earlier suspicions that *pamaquine* (plasmochin) prevents or, given with quinine, cures vivax malaria. The toxicity of pama-

[63] Berliner *et al.*: Jour. Clin. Invest., 1948, 27:98.

quine prohibits general use, but the related compounds *pentaquine* and *iso-pentaquine* are as effective and considerably less hazardous.[64, 65]

Epidemiology and Control. Malaria is the most common infectious disease of man, occurring throughout the warmer regions of the world and extending well into the temperate zones to occupy a large part of the land area between 60° N. and 40° S. latitude. The principal factor in distribution of the infection is climate, which affects both the distribution and abundance of the mosquito hosts and the development of the parasite in the mosquito. The human malarial parasites are not found in lower animals. Man is, therefore, the only reservoir of infection. Racial immunity is known in the case of *P. vivax*. Negroes are less readily infected with most strains than whites and seldom show symptoms. Local populations with high natural immunity to *P. falciparum* have also been reported. Age resistance is not known, but in endemic regions clinical malaria may be rare in native adults who have been constantly reinfected with the local strains of parasites.

Barring rare congenital cases, blood transfusions and accidental transfers of blood, as by drug addicts sharing contaminated syringes, human malaria is transmitted exclusively by the bites of certain mosquitoes of the genus Anopheles. This genus, comprising some two hundred species throughout the world, is readily distinguished from the common house mosquitoes of the genera Culex and Aedes by several characteristics (Fig. 213). The adult generally rests at an angle to the surface, with proboscis, head and body in a straight line, whereas the others rest parallel, with the head and proboscis turned down. The wings are usually spotted, those of other mosquitoes being unmarked. The palps of the female are as long as the proboscis, giving the impression of three long appendages from the head in addition to the antennae, whereas the other genera have short, barely noticeable palps. The eggs bear inflated floats and are laid singly on the water, whereas in Culex they are laid in rafts on the water and in Aedes they are deposited singly on damp surfaces. The larvae of Anopheles lie flat at the surface of the water, feeding on floating particles, while those of other genera hang down in the water from an elongated breathing tube and usually feed on the bottom.

Only the female mosquitoes feed on blood, most Anopheles biting at dusk or in the night. The breeding places vary greatly in character, almost any type of water collection serving to support the larvae of one or more species of Anopheles. Most species, however, are quite specific in the types of water collections they choose. The principal malarial vector in the southeastern United States, *A. quadrimaculatus*, breeds chiefly around the debris- and weed-covered edges of swamps, ponds and sluggish streams. Important malarial vectors, however, are found in hill streams (*A. minimus* in Southern Asia), brackish marshes (*A. maculipennis* in southern Europe), small temporary pools (*A. gambiae* in Africa), water held in plants growing in the tops of trees (*A. bellator* in Trinidad), etc.

Not all species of Anopheles serve equally efficiently as vectors of malaria, about seventy-five being considered dangerous carriers in one or more regions.

[64] Jones *et al.*: Jour. Clin. Invest., 1948, 27:6.
[65] Alving *et al.*: Jour. Clin. Invest., 1948, 27:35.

The chief determining factors are abundance, contact with man and suscepti-
bility. The last varies greatly among species and among populations within
species. Little is known of the factors in human malaria, but simple genetic
differences have been shown to determine susceptibility of some Culex
mosquitoes to avian malaria.[66] In most situations which have been adequately
studied, contact with man is the principal factor in the importance of species
of Anopheles. Several susceptible species are abundant in the southeastern
United States, but in most of this area only one, *A. quadrimaculatus,* shows
sufficient preference for human blood to serve as an important carrier. In
Europe races[67] of *A. maculipennis,* distinguishable only by egg patterns, differ
so greatly in their relative preferences for human and animal blood that some
are dangerous vectors while others, equally susceptible, are insignificant in the
spread of malaria.[68]

Malaria control rests largely on the interruption of the mosquito phase of the
life-cycle. This may be accomplished by reduction in mosquito numbers or
prevention of contact between mosquitoes and man. If mosquito reduction is
to be attempted, the differences in relative importance of different species, or
even of the same species in different regions, require that the principal vectors
in a given area be determined before control is attempted. Such determina-
tion is of value in avoiding waste of resources on unimportant mosquitoes.
Furthermore, unplanned control efforts may completely miss the principal
vectors and have even, on occasion, made matters worse by favoring the breed-
ing of dangerous species. The "natural index" of infection, determined by
examining stomachs and salivary glands of wild-caught female mosquitoes for
oocysts and sporozoites of malarial parasites, is the best guide to relative im-
portance of a species. Unfortunately, the figure is often so low that dissection
of large numbers of mosquitoes is required. In such cases expediency may
justify indirect measures. Susceptibility may be determined by exposure of
laboratory-reared mosquitoes to carriers and dissection of the fed females for
stages of the parasite ("experimental index"). Contact with man may be meas-
ured by the location of resting mosquitoes (in houses as against stables) or by
precipitin tests on fed mosquitoes to determine the type of blood they contain.

If control is to be applied to the breeding of mosquitoes, the habits of the
important vectors in an area must be determined. Elimination of breeding places
is the method of choice in many regions. Water collections may be destroyed
by drainage or filling. Salinity may be controlled by tide-gates. Breeding
around the margins of reservoirs may be eliminated by water-level fluctuations.
Shade may be increased or decreased by control of vegetation. Streams may be
cleared or periodically sluiced. Specific methods, it will be obvious, depend on
local conditions and the habits of the mosquitoes involved. Several effective
poisons (larvicides) are available for attack on the aquatic stages. Oils sprayed
on the water surface are toxic to the eggs, larvae and pupae. Paris green and
the newly rediscovered DDT kill larvae which ingest them. A minor method
of local value is the stocking of ponds and pools with top-feeding minnows,
Gambusia or Lebistes, which eat mosquito larvae.

Attack on adult mosquitoes, formerly a secondary measure, has re-

[66] Huff: Jour. Prev. Med., 1931, 5:249.
[67] These are now designated as separate species by many authors.
[68] Hackett: *Malaria in Europe.* Oxford University Press, London. 1937.

cently assumed major significance. Spray-killing of adults with pyrethrum in oil or especially with pyrethrum-freon aerosols is of value. Even swatting has its place. Screening, bed-nets, protective clothing, repellents and the avoidance of evening exposure outdoors are all useful and have contributed to the control of malaria. The new insecticides, DDT and gammexane and their relatives, have given malariologists a weapon of tremendous value. Sprayed or painted on walls, screens, etc., they kill adult mosquitoes which rest on these surfaces, even several months after application. For those species of Anopheles which enter houses to bite man these insecticides are already demonstrating their value as an inexpensive and highly effective tool in malaria control.
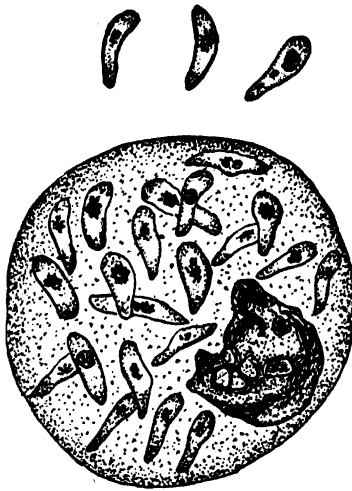
Fig. 214. *Toxoplasma*, × 3500 (Redrawn from Chatton and Blanc).

Malarial Parasites of Lower Animals. Malarial parasites fundamentally like those of man occur in apes, monkeys, bats, rodents, birds and lizards. Since lower animal hosts are not available for the human malarial parasites, the natural parasites of monkeys and birds have been widely used experimentally. Among the important discoveries made in these infections are the mosquito transmission of malaria, the chemotherapeutic activity of quinacrine, paludrine, chloroquine, pamaquine, pentaquine and isopentaquine, exoerythrocytic and cryptozoic stages and much of the information on immunity. The species most widely studied are *P. brasilianum*, *P. knowlesi* and *P. cynomolgi* of monkeys and *P. cathemerium*, *P. relictum*, *P. gallinaceum* and *P. lophurae* of birds.

PARASITES OF UNCERTAIN AFFINITIES

Toxoplasma. Parasites known as Toxoplasma were discovered in 1908 in rodents by Nicolle and Manceaux in Africa and by Splendore in Brazil.[69] They are crescentic or oval bodies, 6 to 7 by 2 to 4 microns in size, staining blue with a red nucleus in Giemsa preparations (Fig. 214). They reproduce only in cells, chiefly monocytes and endothelial cells, dividing by binary fission,

[69] *Cf.* Sabin: Jour. Amer. Med. Assn., 1941, *116*:801.

but are often found free in body fluids. Many mammals and birds are susceptible, and infection has been produced by a variety of routes, though the natural means of spread are unknown. Artificial cultivation has not been achieved. Toxoplasma have been reported in several cases of encephalitis, chiefly in newborn infants and children[69] and in a typhus-like pneumonitis of adults.[70] While they are commonly called sporozoa, it must be said that their relationships are unknown, and it is not even certain that they are protozoa.

Sarcocystis. In the muscle fibers of various vertebrates, particularly ruminants and rodents, are seen long, cylindrical tubes containing numerous crescent-shaped "spores." They are called "Miescher's tubes" for their discoverer.[71] While these organisms, known as Sarcocystis, have long been classed with the sporozoa or cnidosporidia, recent studies indicate that they are actually fungi.[72]

THE METAZOA

All phyla of animals other than protozoa are commonly designated by the term *Metazoa,* and it will be convenient to mention certain general characteristics of the human parasites, mostly known as "worms," which belong to this aggregate. They are usually macroscopic in size, ranging from about a millimeter to several meters in length. Their structure is generally complex, and their life-cycles vary from the simple production of infective eggs or larvae to complex alternation of generations involving as many as three different hosts. An important general characteristic is that multiplication usually does not occur in the human body, so that infection does not increase in intensity in the absence of reexposure. Successful cultivation of parasitic worms has not been achieved. However, some species have been maintained *in vitro,* sometimes with partial growth, for periods of as long as several months.[73] The great variety of metazoan parasites of man makes it impossible to discuss them extensively in a small space. Therefore, representative examples will be used wherever possible. For fuller discussions the reader is referred to Faust's text[74] and to the general texts mentioned earlier (p. 754).

PLATYHELMINTHES

The phylum *Platyhelminthes,* or "flatworms," is differentiated from other phyla of animals by several characteristics. They are bilaterally symmetrical and composed of the three primitive germ layers of tissues—ectoderm, endoderm and mesoderm. The body cavity is not lined with mesoderm, as in higher forms, but filled with a spongy mass of cells, the *parenchyma.* The digestive tract is absent or, if present, lacks an anus, solid wastes being regurgitated through the mouth. There are three major classes— the Turbellaria, which are free-living forms or external parasites of aquatic animals, exemplified by Planaria; the Trematoda, or "flukes," which are all parasitic; and the Cestoidea, or "tapeworms," also all parasitic.

[70] Pinkerton and Henderson: Jour. Amer. Med. Assn., 1941, *116*:807.
[71] Miescher: Ber. v. d. Verhandl. Naturf. Ges. Basel, 1843, 5:198.
[72] Spindler and Zimmerman: Jour. Parasit., 1945, *31,* Suppl.: 13.
[73] *Cf.* Hoepple, Feng and Cho: Chin. Med. Jour., 1938, 2 Suppl.: 243; Hobson: Parasit., 1948, 38:183.
[74] Faust: *Human Helminthology,* Lea & Febiger, Philadelphia. 1948.

The human lung fluke, *Paragominus westermani*, and the blood fluke, *Schistosoma mansoni*, will serve as examples of the trematodes.

Paragonimus Westermani. The adult worm, found by Westerman in 1877 in the lungs of a tiger, was described by Kerbert[76] in 1878. Nakagawa, Yokagawa and others elucidated the life-cycle.[77] Several species have been described, but it is not certain that they are valid, and some authors believe that there is only one species, *Paragonimus westermani*.[77]

Characteristics and Life-Cycle. The adult lung fluke is an ovoid reddish worm, 7 to 12 mm. long by 3 to 6 mm. in diameter, covered with a transparent cuticle which is studded with spines. The anatomy of a flattened specimen is shown in Fig. 212. Two muscular suckers are present, one on the ventral surface, the *acetabulum*, the other at the anterior end, the *anterior sucker*, perforated by the mouth. The mouth opens into a muscular pharynx, followed by a thin esophagus. This divides into two blind *intestinal ceca* extending down the sides of the body. The nervous system is simple, and no special sense organs are present. The excretory system consists of a bladder opening at the posterior end and receiving collecting tubules which extend throughout the body, terminating in characteristic "flame cells." The circulatory system is rudimentary, consisting of indefinite channels through the parenchyma.

The adult fluke is hermaphroditic, both male and female reproductive systems occurring in the same individual. These systems are typically elaborate and, as in most flukes, are the most prominent structures of the body. In the female system a single ovary connects via an oviduct with the *oötype*, where the eggs are formed and receive their shells. In its course the oviduct receives the common *vitelline duct*, into which shell material comes from the branched *vitellaria*, glands occupying the sides of the dorsal surface of the body. The oviduct also has a diverticulum, the *seminal receptacle*, in which sperms are stored.[78] An inconspicuous duct of unknown function, *Laurer's canal*, runs from the oviduct to an opening on the dorsal surface of the body. The oötype, surrounded by gland cells, *Mehlis' gland*, opens into the uterus, a long coiled tube which carries the completed eggs to the common genital pore near the acetabulum.

The male reproductive system consists of paired testes in the posterior part of the body, connecting by vasa efferentia with the vas deferens, which empties into the common genital pore along with the uterus. Part of the vas deferens is widened into a sperm reservoir, the seminal vesicle, and a more distal portion is the glandular *prostatic region*. The terminal portion forms a muscular copulatory organ, the *cirrus*.[79]

The eggs produced by adult worms in the lung are coughed up and either

[75] For a general account of the biology of the trematodes cf. Dawes: *The Trematoda*. The University Press, Cambridge, England. 1946.

[76] Kerbert: Zool. Anz., 1878, *1*:271.

[77] Cf. Ameel: Amer. Jour. Hyg., 1934, *19*:279.

[78] In Paragonimus the seminal receptacle is small, though in some human flukes it is a prominent structure.

[79] These three organs are contained in the *cirrus sac* in most forms, but this is absent in Paragonimus.

m
ph
int
vit
ac
gp
ov
sc
vdef
vitd
t
exc

adult

metacercaria

MAN CRAB

egg

WATER

miracidium

SNAIL

cercaria

sporocyst

redia II

redia I

Fig. 215.

E. Hanford
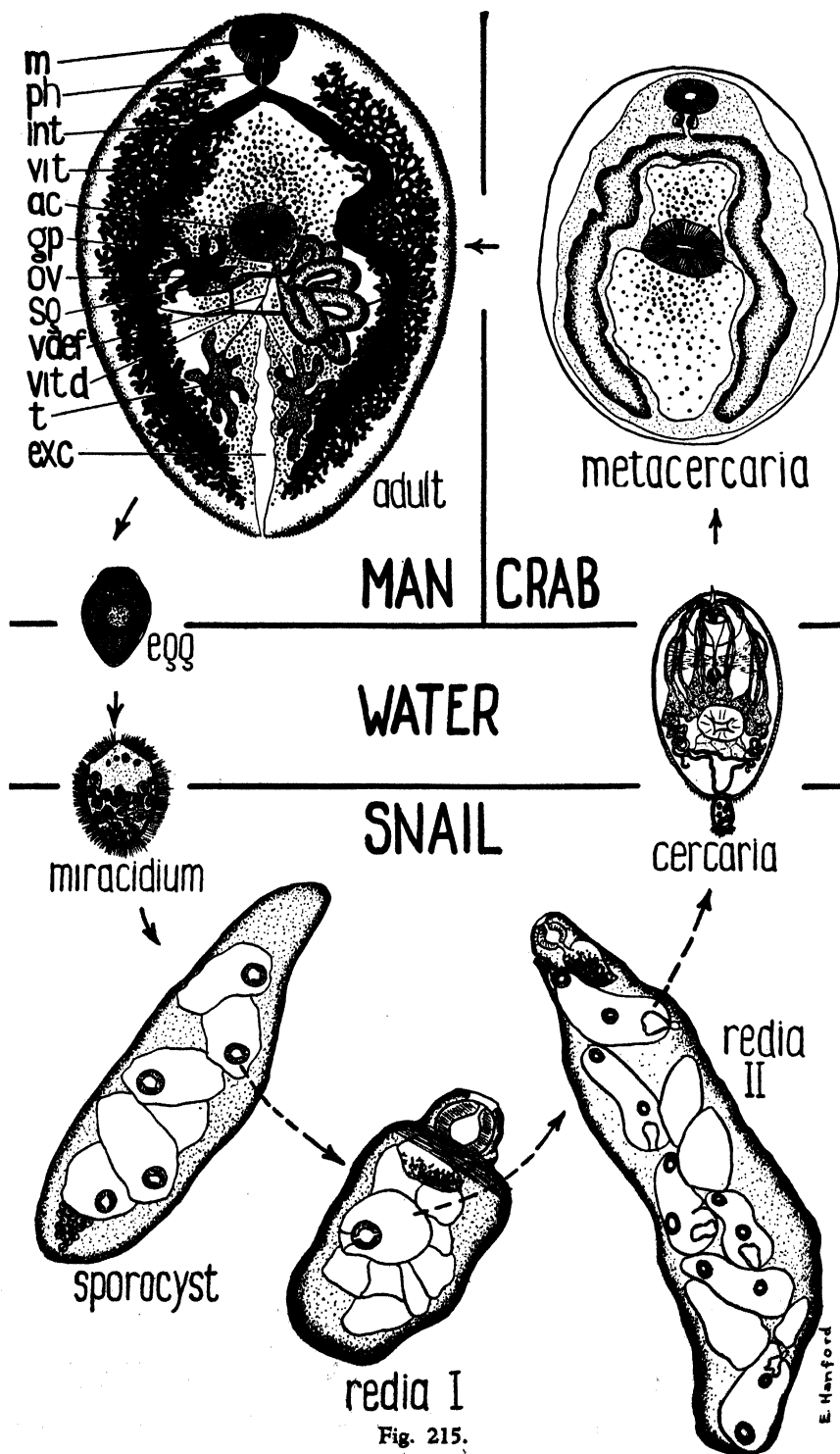
escape in the sputum or are swallowed and passed in the feces. They are ovoid, averaging 60 by 90 μ in size, and show a removable cap, the *operculum*, at one end. The embryos are undeveloped at the time of escape from the host. They develop in from two to six weeks in water, where the eggs hatch by the opening of the operculum to release a free-swimming ciliated larva, the *miracidium*. This larva survives only a few hours unless it succeeds in penetrating the tissues of a suitable intermediate host. Various snails of the genera Melania and Pomatiopsis can serve as hosts. In the lymph spaces of the snail the miracidium becomes an irregular, thin-walled sac, the *sporocyst*, growing to a final length of about 0.4 mm. Cell masses within the sporocyst enlarge, developing in about one month into twelve or more *rediae*. These escape by rupture of the sporocyst and grow to a length of about 0.3 mm. They differ from the sporocyst principally in having a rudimentary digestive tract and a *birth pore*. By a reproductive process similar to that in the sporocyst the first generation redia produces twelve or more second generation rediae. These escape by way of the birth pore and grow to a length of about 0.5 mm. Within each second generation redia arise twenty or more tailed larvae, or *cercariae*. The cercaria is essentially a rudimentary adult worm. It differs principally in having a small tail and two types of penetration organs, a stylet in the mouth region and several gland cells, opening at the anterior end, which secrete histolytic enzymes. About three months after entry of the miracidium the cercaria escapes from the snail and moves about in the water, dying in one or two days unless it finds a suitable second intermediate host, a crayfish or crab of any of various genera, especially Astacus and Potamon.

The cercaria penetrates the softer part of the integument of a crab, loses its tail and grows in the tissues into the *metacercaria*, a near-spherical body about 0.4 mm. in diameter enclosed in a cyst wall. The full development requires about one month, after which the metacercaria is infective when eaten by man. In the small intestine the cyst wall softens, releasing the metacercaria, which penetrates the wall of the small intestine and reaches the abdominal cavity within a few hours. It wanders rather aimlessly but usually passes through the diaphragm within a few days and invades the lung, where it becomes encapsulated by the tissues and grows to the adult stage. After about six weeks eggs may be found in the sputum.

Paragonimiasis. The adult worms in the lung, and particularly the eggs they produce, cause tissue destruction, inflammation and hemorrhage. Local pneumonic processes with cough and bloody sputum are characteristic. As in most worm infections, the damage is roughly proportional to the number of organisms present. In severe cases weakness and even death may result from the extensive lung injury. Aberrant worms in other tissues, as in the brain, cause local effects.

There is no evidence concerning acquired *immunity* to infection. Human infections have been shown to persist at least six years in the absence of reexposure, but it is said that they often clear up after several years.

Fig. 215. Life-cycle and morphology of Paragonimus. (Larval stages redrawn from Ameel, 1934, $\times$ 120. Adult worm $\times$ 7.) m, mouth; ph, pharynx int, intestinal cecum; vit, vitellaria; ac, acetabulum; gp, genital pore; ov, ovary; sg, oötype; vdef, vas deferens; vitd, vitelline duct; t, testis; exc, excretory bladder.

Diagnosis of lung fluke infection depends on the finding and identification of characteristic eggs in the sputum or feces. As in other trematode infections, the size and structure of the eggs (see Fig. 215) are diagnostic. Other trematode eggs may occur in the feces, but only those of Paragonimus normally occur in the sputum.

Epidemiology and Control. Various mammals are susceptible to Paragonimus infection. Cats, dogs, mink, muskrats and man are the usual natural hosts. Infection in lower animals is known in Asia, Africa, North and South America,
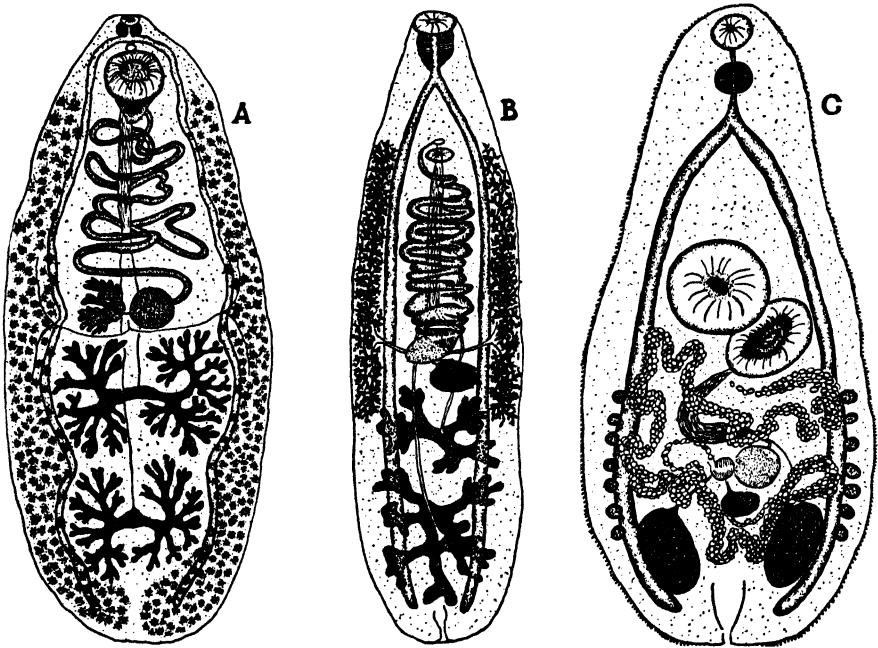
Fig. 216. Trematode parasites of man. Note differences in magnification. A, Fasciolopsis buski; × 3. B, Clonorchis sinensis; × 5. C, Heterophyes heterophyes, × 60. (Adapted from various sources.)

and the United States. Human infection is limited by food habits, occurring commonly only in the Far East. The metacercariae in infected crustacea are destroyed only by thorough cooking or considerable exposure to pickling sauces, and in the endemic areas fresh water crabs, raw or slightly pickled, are considered a delicacy. It should be emphasized that the asexual reproduction in snails is obligate and that only the metacercariae and possibly the cercariae are infective for mammals. Thus, direct transfer from mammal to mammal does not occur, and numerical increase of an individual infection is unknown in the absence of reexposure.

Chemotherapy of human infections is useless for control both because of its unreliability and because lower animals are an important reservoir. Destruction of snails has generally failed. The only effective control measure known is education to the dangers of eating improperly cooked fresh-water crustacea.

It should be noted here that while such modification of food habits is the best control method for all human trematode infections except the blood flukes, established habits and prejudices often interpose difficulties in its execution.

Fasciolopsis Buski. The large adults of this species attain a length of 7 cm. (Fig. 216). They are found attached to the mucosa of the small intestine of man and the pig. The infection is most common in China but occurs in other parts of Asia. The large eggs (average 140 by 80 μ, see Fig. 217) escape in the feces and develop in water, where the miracidia invade snails of the genera Segmentina and Planorbis. The development in the snail, like that of Paragonimus, comprises a sporocyst generation and two generations of rediae. The long-tailed cercariae encyst on aquatic plants, particularly the water chestnut, as metacercariae. These plants, eaten raw or peeled with the teeth,
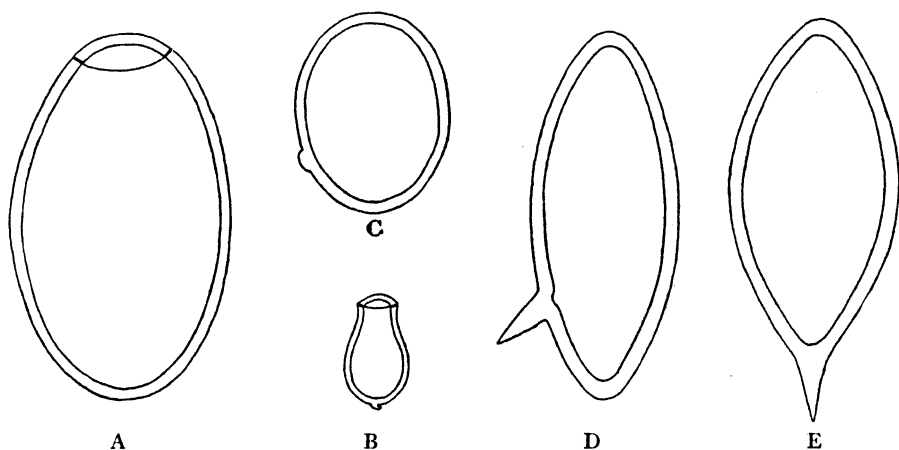
Fig. 217. Shells of some trematode eggs; × 300. *A, Fasciolopsis buski. B, Clonorchis sinensis (Heterophyes heterophyes* is very similar). *C, Schistosoma japonicum. D, S. mansoni. E, S. haematobium.*

convey the infection to man or the pig. Symptoms are generally related to the intensity of infection. They consist of intestinal disturbances and generalized edema. The latter has been attributed to toxemia but may be merely nutritional, resulting from the food-robbing of the host by the worms. Severe infections may cause death. As in many other intestinal worm infections, β-naphthol, carbon tetrachloride and especially hexylresorcinol are efficient drugs. Control is achieved by treatment of human infections, proper sewage disposal, and education to the dangers of eating raw plants from contaminated water. The problem is complicated by the practice, common in endemic areas, of using human feces as fertilizer.

The closely related *Fasciola hepatica* infects the livers of sheep and cattle, causing "liver rot," and is of great economic importance. It is important also because it was the first trematode whose life-cycle was worked out. Occasional human cases are known. *Fasciola gigantea* and *Fascioloides magna* of herbivores also occasionally infect man. Another economically important parasite of the liver of herbivores, *Dicrocoelium dendriticum,* has been found rarely in man.

Heterophyes Heterophyes. *Heterophyes heterophyes* is a small trematode, about 1.5 mm. long, of the intestine of man, cats, dogs and other fish-eating mammals in Egypt, Asia Minor and Asia (Fig. 216). The eggs are small, averaging 25 by 16 μ (Fig. 217). Metacercariae are ingested by man in insufficiently cooked or salted fish. The adult trematodes produce only minor gastro-intestinal disturbances, but it is reported that the eggs are often deposited deep in the mucosa, whence they reach the general circulation and are localized in various distant tissues.[80] The inflammation in these tissues, particularly the myocardium and brain, is said occasionally to cause serious symptoms or death. Several related species attack man. The most important of these is *Metagonimus yokagawai*, which occurs in man and other fish-eating animals in the Balkans, Palestine and Asia.

Other Intestinal Trematodes. Several species of Echinostoma, Echinochasmus and Euparyphium occasionally parasitize man. They are elongate trematodes with a ring of large spines on the head region. The human infection is acquired by ingestion of raw snails or mussels containing the metacercariae. Various mammals and birds are the normal hosts of the adult worms. Occasional human infections are also reported with *Gastrodiscus hominis* and *Watsonius watsoni*. Their life-cycles are unknown, but since they chiefly parasitize herbivorous mammals it is presumed that the metacercariae are found on plants.

Clonorchis Sinensis. The Chinese liver fluke, *Clonorchis sinensis*, is a thin, elongated trematode, 1 to 2 cm. in length (Fig. 216). The adult worm inhabits the smaller bile ducts of man, cats, dogs and other fish-eating mammals in China and Japan. The eggs (Fig. 217), averaging 29 by 17 μ, pass out with the bile and escape in the feces. They are fully developed but do not hatch in the water. Upon ingestion by suitable snails, usually of the genera Parafossalurus and Bithynia, they hatch, and the miracidia become sporocysts in the lymph spaces. Rediae produced in the sporocyst give rise to long-tailed cercariae. These encyst as metacercariae in the tissues and on the skin and scales of various fresh-water fish. Ingested metacercariae hatch in the small intestine, and the young worms migrate up the bile ducts to develop into adults three to four weeks later. Thorough cooking (100° C. for fifteen minutes) is necessary to destroy the metacercariae. Infection of man occurs by ingestion of the metacercariae, possibly in drinking water or by contamination of the fingers in handling infected fish, but usually by consumption of insufficiently pickled or cooked fish, a favorite article of diet in the endemic regions. Hazards are increased in these areas by the practice of propagating fish for market in ponds fertilized with human feces.

Human infection is characterized by proliferation of the bile-duct epithelium and of the surrounding connective tissue. This results in liver cirrhosis, destroying liver parenchyma and obstructing portal blood flow. Intestinal disturbances, liver enlargement and ascites are the common symptoms, severe infections resulting in death. No reliable drugs are known. Control, as in the trematode infections discussed above, depends principally on education concerning avoidance of undercooked infected food, in this case fresh-water fish.

Related species, particularly *Opisthorchis felineus* and *O. tenuicollis*, infect

[80] Africa, DeLeon and Garcia: Acta Med. Philippina, Monogr. Ser., 1940, *1*.

fish-eating mammals and, less commonly, man in parts of Central Europe and Asia.

The Human Blood Flukes. Bilharz[81] found trematodes in the mesenteric veins of an Egyptian patient. Three species are now known to cause schistosomiasis or, as it is called in much of the medical literature, "bilharziasis" in man. *Schistosoma mansoni* is taken here as an example.

Morphology and Life-Cycle. Unlike the trematodes discussed above, the adults of *Schistosoma mansoni* are unisexual, male and female reproductive systems occurring in separate individual worms. The adults are found together in the smaller mesenteric veins, the long, thin female enclosed in a groove on
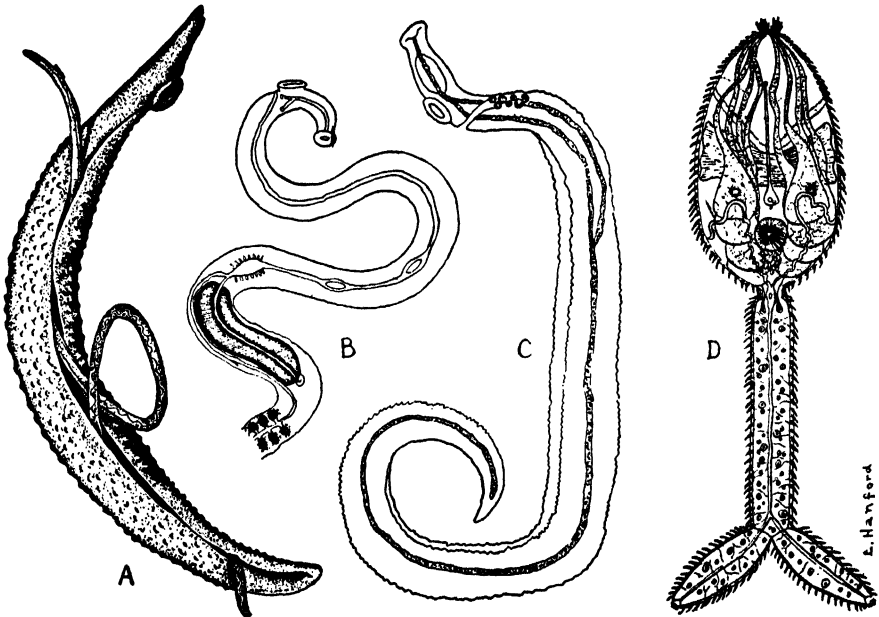
Fig. 218. *Schistosoma mansoni.* Note differences in magnification. *A,* Adult worms; × 8. *B,* Anterior end of female worm; × 20. *C,* Adult male; × 10. *D,* Cercaria; × 180. (*A* and *C* redrawn from Manson-Bahr. *D* redrawn from Cort.)

the ventral side of the male (Fig. 218). The cylindrical female is 1 to 1.5 cm. in length by about 0.25 mm. in diameter. An anterior sucker surrounds the mouth and shortly behind it is a stalked acetabulum. The intestinal ceca, unlike those of Paragonimus, unite before the middle of the body, a single tube continuing to the posterior end. The oval ovary lies immediately anterior to the union of the intestinal ceca, and the uterus, containing eggs, extends forward to open behind the acetabulum. The posterior half of the body is occupied by vitellaria. The male is about 1 cm. long by about 1 mm. in diameter, its integument covered with coarse tubercles. The suckers and alimentary tract are similar to those of the female. The reproductive system consists of a cluster of eight or nine round testes in the anterior region emptying into a seminal vesicle, which opens to the outside just behind the ventral sucker.

[81] Bilharz: Zeits. f. Wiss. Zool., 1852, 4:72.

The large eggs, averaging 150 by 65 μ, have no operculum but show a prominent spine at the side (Fig. 217). They are deposited in the fine venules of the mesenteric veins in the wall of the intestine, where they lodge, held by the spine in the distended vessels. Stasis of the blood, injury by the spine and enzymes secreted by the developing embryo combine to rupture the vessel, allowing the eggs to escape into the tissues and eventually into the lumen of the intestine. They are mature at the time they leave the body in the feces. On dilution of the feces with water, the shell ruptures, releasing the miracidium. In suitable snails of the genera Planorbis and Physopsis there are two generations of sporocysts resulting, after about six weeks, in the production of cercariae with long, forked tails (Fig. 218). Escaping into the water the cercariae swim actively or rest at the surface, dying in about three days if they do not reach a susceptible final host. Coming in contact with human skin they attach and, as the surface film of water is drying, actively penetrate the skin. The tail is lost and the parasite is carried via lymph and blood to the portal veins in the liver. Here maturation occurs to the adult forms, which mate and migrate distally in the portal system to the mesenteric veins. Four to eight weeks after infection the sexually mature worms begin egg-production. The infection may persist for many years without reexposure.

The other species of human blood flukes differ from *Schistosoma mansoni* in size, details of morphology, egg structure, location in the definitive hosts, types of snail hosts and geographical distribution. *Schistosoma haematobium* adults occur predominantly in the venules of the urinary bladder wall, the eggs escaping in the urine. The eggs are large, 150 by 60 μ, with a terminal spine (Fig. 217). The male, 1 to 1.5 cm. long, is covered with fine tuberculations and has four large testes. The female is about 2 cm. long. Snails of the genera Planorbis, Physopsis and Bulinus serve as intermediate hosts.

Schistosoma japonicum occurs in the venules of the intestine. The eggs average 80 by 65 μ, with a small lateral knob rather than a spine (Fig. 217). They escape in the feces, the miracidium invading snails of the genera Oncomelania and Hemibia. The male is 1 to 2 cm. long, with a smooth integument, and has seven or eight testes. The female is 1 to 2.5 cm. long.

Schistosomiasis. Penetration of the skin by the cercariae may cause a mild dermatitis. In the case of the human blood flukes this is not so prominent as in that of several species normally parasitic in lower animals. In parts of the northern United States and of Europe, these species cause a severe schistosome dermatitis or "swimmer's itch." There is no evidence that they mature in man.

The important disease processes of human schistosomiasis are the result of tissue destruction by the eggs. In the case of *Schistosoma mansoni* this results in dysentery and later in benign tumors, constrictions or fistulae of the lower intestine. Eggs carried to the liver arouse inflammation with enlargement and cirrhosis resulting in ascites. Enlargement of the spleen is common. *Schistosoma japonicum* produces a similar picture, the liver pathology generally predominating. In infection with *Schistosoma haematobium* the worms and eggs in the wall of the bladder cause hematuria, pain, chronic inflammation, calculi, benign tumors and fistulae. Severe cases show grave injury to the entire urinary tract. In all blood fluke infections the benign tumors may become malig-

nant. In Egypt epidemiologic evidence suggests that *S. haematobium* is an important factor in the etiology of carcinoma of the bladder.

IMMUNITY. Information is lacking on human immunity to blood fluke infections, though it is known that infections are most severe in children and uncommon in late adult life. Animals cured of an initial infection show increased resistance to reinfection. Antibodies are demonstrable with extracts of adult worms or heavily infected snail livers. That the reactions are group-specific is shown by the fact that extracts of *Fasciola hepatica* react with sera of individuals infected with the schistosomes. The precipitin test is unreliable, but complement fixation is a dependable tool for diagnosis. A skin test is useful but remains positive long after cure.

DIAGNOSIS. Laboratory diagnosis preferably is based on the finding of characteristic eggs in urine, feces or rectal scrapings. Early in infection or after serious tissue damage they may not be detectable, and in such circumstances the immunological tests are of great value. When eggs are not demonstrable in direct smears, the acid-ether concentration technique is very useful.[82]

Antimony compounds, particularly sodium antimony tartrate, are effective in killing the adult worms.

Epidemiology and Control. Blood fluke infections may be acquired from drinking water, the cercariae penetrating the mucosa of the upper alimentary tract. Occasional infections are also known in newborn infants whose mothers were exposed to infection during gestation. The chief means of infection, however, is by contact of the skin with water containing the cercariae. This occurs especially in the working of rice fields fertilized with human feces, but also in bathing and chance contact. Man is the important reservoir of *Schistosoma mansoni* and *Schistosoma haematobium*, though both are found naturally in monkeys, and other laboratory animals can be infected. *S. japonicum* occurs naturally in a wide variety of animals, including dogs, cats, pigs and cattle. *S. mansoni* is found in Egypt and Central Africa, the West Indies and northern South America, *S. haematobium* in a large part of Africa and in Asia Minor, *S. japonicum* only in the Far East.

Control of blood fluke infections depends on preventing the access of eggs to water containing suitable snails or avoidance of contact with water containing the cercariae. Little success has been attained. Safe sewage disposal or storage of sewage which is to be used for fertilizer is of value. Local destruction of snails is possible with lime or copper salts. Periodic drying of irrigation ditches is also effective except in the Far East, where the snail hosts can withstand long periods of drying. Repellents, such as benzyl benzoate, impregnated in clothing or applied to the skin in ointments discourage penetration by the cercariae. They cannot, however, be expected to prove useful in general control of the disease. Ordinary chlorination of drinking water kills the cercariae.

Other Species. The lower animal parasites which produce "swimmer's itch" have been mentioned above (p. 792). One of them, *Schistosomatium douthitti*, is of especial interest because it is easily studied in laboratory mice. Several species produce important diseases of domestic animals in Africa and India.

[82] Weller and Dammin: Amer. Jour. Clin. Path., 1945, *15*:496.

CESTOIDEA

The cestoidea, or tapeworms, generally consist of a colony-like chain of flattened segments, each of which is a semi-independent unit with a complete set of reproductive organs. A *scolex* at the anterior end of the chain serves to anchor the worm in place, and the nervous and excretory systems are shared by the whole organism. There is no alimentary tract, soluble food substances being absorbed through the body surface. All are parasitic, the adults usually in the alimentary tract of the definitive host and the larval stages in the tissues of an intermediate host. Two orders of tapeworms, the Pseudophyllidea and Cyclophyllidea, contain human parasites. The Pseudophyllidea are discussed later (see *Diphyllobothrium latum*, p. 800). Most of the tapeworms of man belong to the Cyclophyllidea, of which *Taenia solium* will serve as an example.
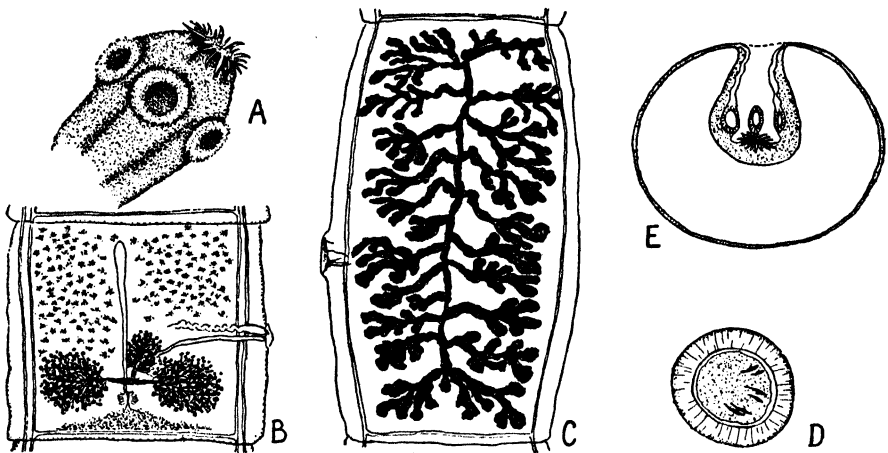
Fig. 219. *Taenia solium. A,* Scolex; × 20. *B,* Mature proglottis; × 5. *C,* Gravid proglottis; × 5. *D,* Egg; × 500. *E,* Cysticercus; × 5 (Adapted from various sources).

Taenia Solium. The pork tapeworm, *Taenia solium,* was known in ancient times. Küchenmeister[83] first suspected its relationship to "bladderworms" in pork and demonstrated their transformation into the adult worms in the intestine of a condemned criminal.

Characteristics and Life-Cycle. In the intestine of man the adult *Taenia solium* attains a length of 2 to 10 meters. Buried in the intestinal mucosa of the host is the scolex, or "head," a rounded cubical organ about 1 mm. in diameter bearing four large, cup-like suckers and a pad, the rostellum, with twenty to thirty-five hooks (Fig. 219). Behind the scolex is a thin neck which merges into a region of proliferation of segments, or *proglottides.* As new segments are produced, the older ones are pushed back, developing sexually, until near the middle of the worm mature proglottides are found. They average 0.5 cm. square. The genital pore occurs on either side of the proglottis, alternating irregularly from segment to segment. The male reproductive system consists of small follicular testes scattered throughout the dorsal part of the segment,

[83] Küchenmeister: Wien. med. Wchnschr., 1855, 5:1.

emptying through vasa efferentia into a coiled vas deferens which ends in the muscular copulatory organ, or cirrus, at the genital pore. In the female system the vagina extends from the genital pore to the oötype in the posterior central part of the segment. On either side of the oötype are ovaries and behind it the vitellaria. A blind uterus extends forward from the oötype.

As the segments move toward the posterior end of the worm, the uterus fills with eggs, becoming branched to fill most of the segment in the *gravid* proglottis, which is about 0.5 x 1 cm. in size. In *T. solium* there are about ten branches on each side of the uterus. The gravid segments become detached singly or in small groups and pass out with the feces. Before or after escape from the body they burst, releasing large numbers of eggs. These eggs, averaging 35 μ in diameter, are nearly spherical and provided with a thick "shell" of characteristic porous structure. Within the shell is seen the *onchosphere,* an embryo bearing six hooks.

Ingested by a suitable intermediate host, the eggs hatch in the intestine and the embryos penetrate the intestinal wall to enter the blood or lymph. They are carried to various parts of the body, developing in the tissues in about two months to the infective stage. Infection is most intense in the muscles, but other tissues also contain the parasites. This stage, a *cysticercus,* consists of a scolex like that of the adult, a short neck, and a fluid-filled sac about 1 cm. in diameter. Because of the sac, this larval stage is commonly called a "bladder-worm." In the tissues the scolex is invaginated into the bladder. In addition to the pig, other animals may harbor the cysticerci. Among them is man, in whom they occur in various tissues, including the brain.

Man acquires the adult tapeworm by ingestion of undercooked pork containing the cysticerci. In the intestine the bladder is digested away, the scolex attaches to the intestinal wall, and the adult worm develops by growth from the neck region. By two months after infection gravid proglottides containing eggs are being passed in the feces. Infection may last for many years.

The Human Disease. Adult pork tapeworms usually occur singly in the intestine of man. They often cause no noticeable symptoms, though general intestinal discomfort may occur and in children nervous disturbances are sometimes seen. The ravenous appetite popularly associated with tapeworm infections is actually uncommon, loss of appetite being more frequently observed. In contrast with infection by the adult worm, tissue infection with the cysticerci is dangerous. The larvae act like benign tumors; growth is slow and terminates naturally with full development. The injury results from pressure and its seriousness, therefore, depends on the location of the larvae. In most muscles and connective tissues they are of no consequence, but larvae in the eye may affect vision. In the brain they may give rise to epilepsy or other manifestations of local pressure.

There is no evidence of acquired *immunity* to *T. solium* in man, though it has been suggested that the rarity of multiple infections in the intestine indicates immunity to superinfection. In individuals harboring the cysticerci, complement-fixing and precipitating antibodies are detectable. They react with extracts of other tapeworms as well as *T. solium.*

Laboratory *diagnosis* of intestinal infection with *T. solium* is based on recovery of eggs or gravid proglottides from the feces. The eggs are not distin-

guishable from those of the beef tapeworm (see below), but the gravid seg-
ments can be identified by the smaller number of uterine branches, about ten
on each side as against more than fifteen in the beef tapeworm. Diagnosis of
the cysticercus infection is rarely made before operation or necropsy, but the
immunological reactions mentioned above have been used. Extract of male
fern is effective in expelling the adult worm. There is no treatment known
for the larval infection except surgery.

Epidemiology and Control. The larvae of T. solium occur most commonly
in pigs but are found also in man, monkeys, sheep, camels and dogs. Before
1850 in Berlin 2 per cent of human autopsies showed the cysticerci, but the
incidence is now much lower. Pigs acquire the infection by contamination of
their food or water with human feces. Larval infections in man are incurred
chiefly by contamination of food, water or fingers with eggs from human feces,
but it is also possible that in the intestine of an individual harboring the adult
worm the eggs may hatch without reaching the outside. Whether or not eggs
must leave the body before they are infective, a person with the adult worm
in his intestine is a constant hazard to himself as well as to others. Man, the
only known host for the adult tapeworm, acquires the infection by ingestion
of undercooked pork, which often contains large numbers of larvae. Freezing
and thorough cooking kill the larvae, but ordinary pickling and smoking are
ineffective. Government meat inspection reveals most infected carcasses and
has been the most effective single control measure. Other control methods are
directed at the prevention of contact between pigs and human feces.

Taenia Saginata. The beef tapeworm, *Taenia saginata,* resembles *T.
solium* in morphology and life-cycle, but several differences are noteworthy.
The scolex is "unarmed," lacking the hooked rostellum of *T. solium*. The adult
worm is usually about 5 meters long, but occasionally attains a length of 25
to 30 meters. The gravid proglottides are distinguished from those of *T. solium*
by a larger number of uterine branches, usually 15 to 20 on each side. Man is
the only known host of the adult. The cysticerci develop in cattle and other
ruminants, human infection resulting chiefly from consumption of under-
cooked beef. Rare cases of human infection with the larvae have been claimed,
but it is probable that they were aberrant cysticerci of *T. solium*.

Echinococcus Granulosus. The adult of *Echinococcus granulosus* in-
habits the intestines of dogs and related species. It is a minute worm 0.25 to
0.5 cm. in length, consisting of an "armed" scolex and three proglottides, one
immature, one mature and one gravid. Large numbers of the adults may
occur in the intestine of an infected dog.

The natural intermediate hosts are sheep, cattle and other ruminants, but
a wide variety of animals are susceptible, including man, in whom the larva
causes a serious disease. This larva is markedly different from those of the beef
and pork tapeworms discussed above. In the viscera of an infected animal it
attains a diameter of about 1 cm. after five to six months. It is infective after
eight months or more but continues to grow, often, after several years, reach-
ing a diameter of more than 20 cm. The larva, known as a "hydatid cyst,"
is a spherical, fluid-filled sac composed of a thick cuticular wall with a thin
germinative epithelium on its inside surface. From this germinative epithelium
are formed two types of structures. Buds may appear and grow into stalked

vesicles, *brood capsules*, on the inner surface of which stalked cysticercus-like "scolices" are produced. These brood capsules may become detached, enlarge and produce brood capsules and scolices within themselves, when they are known as *daughter cysts*. In the large cysts there may thus be produced many thousand infective scolices, each of which can develop into an adult worm if the cyst is eaten by a dog feeding on the viscera of an infected animal. Thus *E. granulosus* differs from *T. solium* chiefly in the fact that multiplication occurs in the larval as well as the adult stage. The predominant site of larval infection is in the liver. The lung is next in importance, and cysts may occur also in practically every other organ.

Hydatid Disease in Man. Echinococcosis, or hydatid disease, is characterized by two general types of manifestations. First, hypersensitivity develops to components of the hydatid fluid and accidental rupture of a cyst may cause serious, even fatal, reactions. Second, and most important, the growing cyst acts like a tumor, the injury resulting from pressure effects and depending on the localization of the cyst. Rupture of a cyst may give rise to new cysts produced from scolices, daughter cysts, or fragments of germinative epithelium. Various abnormalities occur of which the most important are *alveolar* cysts. These are anomalous in structure, consisting of numerous irregular, jelly-filled cavities which grow irregularly and spread throughout the host tissues like a malignant tumor, usually resulting in death of the host. The majority of alveolar cysts and some unilocular cysts are sterile, producing no scolices. *Osseous* cysts, occurring in bone, may weaken the bone by erosion of its structure.

There is no information available concerning acquired *immunity* to the larval infection in man, though in immunized sheep the cysts develop somewhat abnormally. Dogs injected with antigens derived from the larvae are immune to infection with the adult worm.[84] The hypersensitivity mentioned above can be detected by skin tests with extracts of the cyst fluid. This is the most sensitive immunological test, but complement fixation and precipitation are also demonstrable.[85] These procedures are somewhat less dependable for laboratory diagnosis than microscopic examination of aspirated cyst fluid, but the puncture of a hydatid cyst is exceedingly hazardous to the patient.

Epidemiology and Control. The normal life-cycle of *Echinococcus granulosus* involves dogs and sheep or cattle, man being an accidental host of the larva. For this reason, the infection is most common in the great grazing regions of the world, particularly Australia and New Zealand, North and South Africa, Iceland and southern South America. The incidence in cattle and sheep occasionally exceeds 50 per cent. The alveolar cysts are largely confined to central Europe and Siberia, and this has led some investigators to suggest that they are larvae of a different species of worm, *E. multilocularis*. This hypothesis has not been substantiated. It is weakened by the fact that most alveolar hydatids are sterile and cannot, therefore, produce adult worms in the definitive host.

Control of the infection in domestic animals depends chiefly on preventing dogs from eating offal. Possibly infected waste material should be buried or

[84] Turner, Dennis and Berberian: Jour. Egyptian Med. Assn., 1935, *18*:536.
[85] *Cf.* Dennis: Jour. Parasitol., 1937, 23:62.

burned. Prophylaxis of human infection requires measures to reduce the chance of contamination of human food and water by feces of dogs.

Other Tapeworms Related to Taenia Solium. Species of the genus Multiceps infect dogs as adults and sheep and other lower animals as larvae. The larva, known as a *coenurus*, resembles a cysticercus but has a number of scolices attached to a single bladder. In the brains of sheep the larvae produce a fatal disease. Occasional cases have been reported in man.

Taenia pisiformis is a common tapeworm of dogs and cats, the larvae developing in the abdominal cavity of rabbits. *Taenia taeniaeformis* occurs commonly in the intestine of cats. The larvae in the liver of rats and mice are often associated with malignant tumors. This species is of interest for a number of immunological phenomena. Definite sex resistance is shown by the fact that female rats acquire fewer larvae from a given dose of eggs than do males. Castration of females or injection of male sex hormones reduces their natural resistance, while female hormones increase the resistance of the males.[86] Age
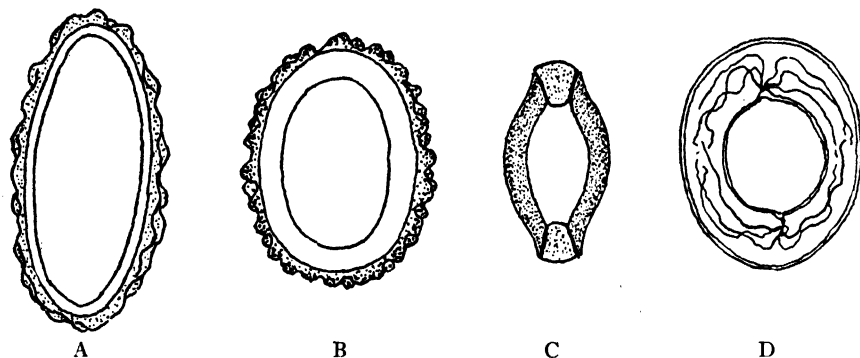
Fig. 220. Shells of some helminth eggs; × about 400. *A, Ascaris lumbricoides* (infertile). *B, Ascaris lumbricoides. C, Trichuris trichiura. D, Hymenolepis nana.*

resistance is also demonstrable in rats. Acquired immunity is induced either by previous infection with the larvae or by immunization with worm material.[87] This immunity is dependent in part on antibodies transferable with serum. Two antibodies are detectable. One acting early in the infection prevents development of the larvae; it is elicited by both infection and vaccination and is removed from the serum by absorption with worm substance. The other acts late in the infection, aborting the development of the larvae in the liver; it is elicited by infection but not by artificial immunization and is not absorbable with parasite material.[88]

Hymenolepis Nana. The human dwarf tapeworm, *Hymenolepis nana,* is a small worm, 1 to 4 cm. in length with the mature proglottides 0.5 to 1 mm. in breadth. The scolex is similar to that of *T. solium* but smaller, about 0.25 mm. in diameter. The mature proglottides are markedly different in appearance from those of *T. solium*, the width being more than four times the length.

[86] Campbell and Melcher: Jour. Infect. Dis., 1940, 66:184.
[87] Miller: Proc. Soc. Exp. Biol. & Med., 1930, 27:926; *ibid.,* 1931, 28:467.
[88] Campbell: Jour. Immunol., 1938, 35:205.

Three large testes occur in each segment. The uterus in the gravid segments is a large, irregular sac. The gravid segments are usually destroyed in the intestine, releasing the eggs, which are found in the feces.

H. nana is unique among human tapeworms in not requiring an intermediate host. Ingested eggs (Fig. 220) hatch in the intestine of man to release an embryo which invades the tissues of an intestinal villus. Here, in about four days, it develops into a *cysticercoid*, a larva with a small bladder and a solid tail. This larva breaks out of the intestinal wall, attaches to the mucosa, and develops into the adult worm. Eggs are found in the feces about one month after infection. It is reported that several types of insects can serve as intermediate hosts, but they are probably unimportant in human spread of the parasite. Large numbers of worms are commonly found in an infected individual, and they may give rise to intestinal and nervous disturbances in children. Male fern extracts eliminate the worms.
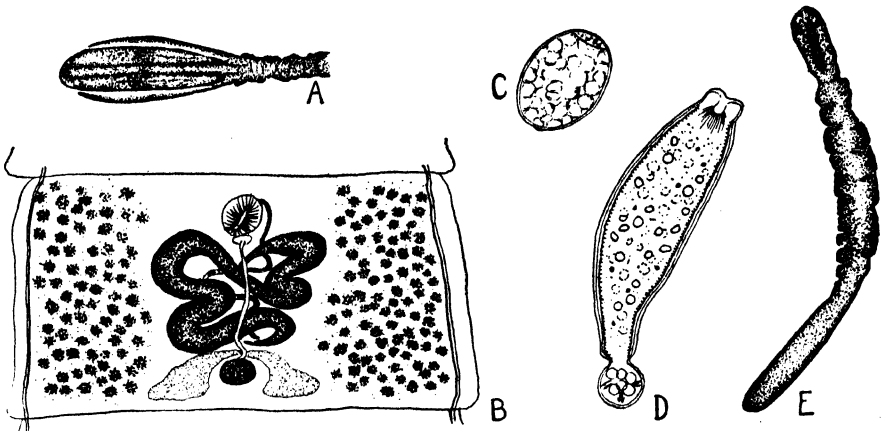
Fig. 221. *Diphyllobothrium latum*. A, Scolex; × 10. B, Mature proglottis; × 6. C, Egg; × 250. D, Procercoid; × 100. E, Sparganum; × 8. (Adapted from various sources.)

H. nana occurs naturally in man, monkeys and rodents. Although the form in rodents has been separated as a distinct species, *H. fraterna*, it is generally considered a variety of *H. nana*.[89] The parasite is cosmopolitan. It is much the most common tapeworm of man in the United States, especially prevalent in children, but occurring in all age groups. Age resistance has not been shown in man but is demonstrable in rats and mice.

The lack of an intermediate host permits direct spread from man to man. It is not known whether the eggs can hatch without having reached the outside. However, an infected individual is particularly liable to reinfection from fingers contaminated with his own feces.

The closely related *H. diminuta* of rats and mice is transmitted by several species of insect intermediate hosts. Occasional human infections have been reported, mostly in children.

Tapeworms of Lower Animals. *Dipylidium caninum,* a common parasite of the intestines of dogs and cats, measures 10 to 50 cm. in length. Each

[89] Shorb: Amer. Jour. Hyg., 1933, 18:74.

elongate, rounded mature proglottis contains two sets of reproductive systems, one opening on each side of the segment. The uterus breaks up into pouches, each containing several eggs. Dog lice and various species of fleas serve as intermediate hosts, the cysticercoids in the insects being infective when ingested. Young children occasionally harbor the adult tapeworm.

Diphyllobothrium Latum. The fish tapeworm *Diphyllobothrium latum* is the most important human parasite in the order Pseudophyllidea. It differs markedly in structure and life-cycle from the species discussed above (Fig. 221). It is a more primitive type, in many respects showing strong resemblances to the trematodes.

Morphology and Life-Cycle. The adult worm in the intestine of man is very large, often exceeding 10 meters in length. The scolex is an elongate ovoid structure, about 1 by 2.5 mm. in dimensions, bearing, instead of the circular suckers of *T. solium*, two elongated grooves which serve as attachment organs. The mature proglottis is broader than long, about 4 by 10 mm. in size. The small testes are scattered throughout the lateral dorsal regions, emptying by fine ducts into the vas deferens. This coiled tube runs from the posterior center of the segment forward to the muscular cirrus, which opens into the ventral genital pore in the center of the anterior region. The vagina extends from the genital pore back to the large oötype in the center of the posterior region. The ovaries lie on either side of the oötype, and the vitellaria are small follicles scattered throughout the lateral ventral regions. Arising from the oötype, the egg-filled uterus coils throughout the center of the proglottis, ending at the uterine pore just behind the genital pore. This permits escape of the eggs without rupture of the proglottis, so that, contrary to the usual situation as seen in *T. solium*, the eggs are released into the intestinal contents and escape in the feces. The spent proglottides at the end of the worm break off and are passed in the feces.

The egg is undeveloped when it escapes in the feces, embryonation requiring a week or more in water. Like the eggs of most trematodes it is ovoid, with an operculum. Average measurements are 65 by 45 μ. Hatching occurs by opening of the operculum to release a free-swimming, spherical, onchosphere covered with long cilia. This embryo, the *coracidium*, dies within a few days unless it is ingested by a suitable small crustacean, one of several species of copepods of the genera Cyclops and Diaptomus. Losing its cilia, the embryo reaches the body-cavity of the copepod and becomes, in two to three weeks, an elongated solid body with a round tail bearing the six hooks seen in the embryo. This stage, the *procercoid*, is about 0.5 mm. long.

If the infected copepod is eaten by a fish, the procercoid penetrates the wall of the intestine and reaches the viscera or muscles. Here, in a week or more, it transforms into the *sparganum*, a worm-like stage 0.5 to 2 cm. long with a rudimentary scolex at the anterior end. This stage, ingested by man in undercooked fish, develops to the adult worm in the intestine, and eggs may be found in the feces after three weeks or more. If the infected fish is eaten by another fish, the sparganum become established in the tissues of the second fish and remain infective for man. Human infections have been known to persist for years.

The Human Disease. D. *latum* commonly produces multiple infections, a

number of worms occurring in the same intestine. The adult worms usually cause no symptoms, though in heavy infections intestinal disturbances may occur, sometimes with generalized edema. A small proportion of infected individuals show severe anemia, indistinguishable from pernicious anemia. It is probable that the infection merely precipitates the condition in susceptible persons. Satisfactory data on acquired immunity are not available. Laboratory diagnosis depends on identification of the eggs in fecal smears. Extract of male fern eliminates the adult worms.

Epidemiology and Control. The adult worm parasitizes man, cats, dogs, bears and other fish-eating mammals, but man is probably the principal source of infection in endemic areas. Susceptible copepods are widespread, and a variety of fish can serve as second intermediate hosts. Endemic centers are known in northern and central Europe, northern Asia, Japan, parts of Africa and the Great Lakes region of North America. Personal protection depends on thorough cooking or freezing of fish from endemic areas and care in handling of such fish. Other control methods are aimed at reducing the pollution of bodies of water with untreated human feces and at the supervision of transportation of possibly infected fish.

Related Species. Several related species of tapeworms, normally parasites of lower animals, are reported occasionally in man. Man also occasionally harbors the spargana of several species of Diphyllobothrium. Infection with these larvae in the subcutaneous tissues, muscles or eye is known as sparganosis. Among the larvae infecting man is the very rare parasite, *Sparganum proliferum,* which multiplies enormously in the subcutaneous tissues and may cause death. The adult worm of this species is unknown. Larval infections with Diphyllobothrium usually result from ingestion of copepods in drinking water. In parts of the Orient split frogs are sometimes used as poultices, and human sparganosis has resulted from the migration of spargana from the frog tissues into the exposed tissues of man.

NEMATODA

All the important human parasites in the phylum Nemathelminthes belong to the class Nematoda. They are cylindrical, elongated worms, unsegmented and with a false body cavity which is not lined with peritoneum as in higher animals. Symmetry is primitively bilateral. Male and female reproductive systems usually occur in separate individuals noticeably different in general appearance. Many are free-living and the parasitic species occur in a great variety of plants and animals.

The structures and life-cycles of the nematodes infecting man are so varied that it will be necessary to concentrate on a few examples. However, the pinworm, *Enterobius vermicularis,* will serve to illustrate the fundamental morphology of the group. A word of caution is necessary here. The order of presentation of species in this section, based primarily on life-cycles, cuts sharply across the lines of zoological classification, separating closely related types and bringing together types of very different structure.[90]

Enterobius Vermicularis. The human pinworm, *Enterobius vermic-*

[90] For a detailed account of the nematodes *cf.* Chitwood and Chitwood: *An Introduction to Nematology.* Monumental Printing Co., Baltimore. 1937.

ularis, normally inhabits the upper large intestine of man, occasionally invad-
ing the female genito-urinary system. The female worm, about 1 cm. long by
0.5 mm. in diameter, is a spindle-shaped organism with a long, pointed tail
(Fig. 222). The outer covering is a smooth, impervious, flexible cuticle, char-
acteristic of nematodes in general. This cuticle, together with the fact that the
body musculature is exclusively longitudinal, gives the worms a characteristic
bending, wriggling type of movement. Among nematodes the cuticle shows
great variety of external structures—knobs, fins, papillae, etc.—which are of
value in technical identification of species. The alimentary tract consists of a
mouth at the anterior end, a muscular esophagus with a posterior bulbous en-
largement, and a thin-walled intestine emptying through the anus near the
posterior end.[91] The female reproductive system empties through a short
vagina a little in front of the middle of the body. Extending forward and
back from the vagina are two separate egg-producing systems (one to several
in other nematodes). Each consists of a broad uterus filled with eggs, followed
by a short narrow oviduct, which terminates in a long, thin ovary coiled about
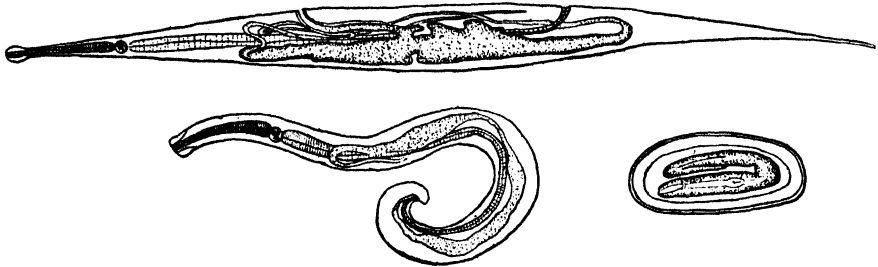
Fig. 222. *Enterobius vermicularis.* Above, female, × 15. Left, adult male, × 15. Right,
egg, × 300. (Redrawn from Faust after Leuckart).

in the body cavity. The male worm is smaller than the female, 2 to 5 mm.
in length and 0.1 to 0.2 mm. in diameter, the posterior end coiled into a tight
spiral and lacking the pinlike tail of the female. The male reproductive system
is a single tube consisting of a testis, a vas deferens, a seminal vesicle and a
muscular ejaculatory duct, provided with glands, which empties with the
intestine into a cloaca. A sharp copulatory spicule can be extruded through the
anus. The posterior end has minute expansions (more prominent in many
other nematodes) to form a clasping *bursa* used in copulation.

Some of the eggs escape in the intestine, passing out in the feces, but usually
the gravid females migrate out of the anus and disintegrate on the surrounding
skin to release their eggs. The eggs average 55 by 25 μ in size, usually show ma-
ture embryos when passed, and are infective immediately. Ingested by man
they hatch in the small intestine, where the worms mature and mate
before passing down to the large intestine. Eggs appear in the feces about two
weeks after infection. The adults in the intestine are short-lived but infection
is maintained or increased in the individual by self-infection and perhaps by
hatching of eggs before escape from the intestine.

[91] Some nematodes, such as *Trichinella spiralis*, have a long non-muscular esophagus
consisting of a thin-walled, narrow tube embedded in a row of characteristic large cells.

Pinworm Infection in Man. The adult worms in the intestine have little or no effect on the host. However, they occur in the vermiform appendix, and since they are said to be more common in diseased than in normal appendices, they have been suspected of an etiologic role in appendicitis.[92] The major symptoms result from disintegration of the females on the perianal skin. The consequent intense itching may induce insomnia and nervousness. Bacterial infection of the inflamed skin may result from scratching. There is no evidence of acquired immunity.

Diagnosis is based on identification of eggs in the feces or, more dependably, in scrapings from the perianal skin. For the latter, the NIH swab, a square of cellophane or Scotch tape examined microscopically after being rubbed over the skin, is particularly useful. Several drugs, particularly gentian violet, hexyl-resorcinol and phenothiazine, are effective in eliminating the worms.

Epidemiology and Control. Man is the only host of *E. vermicularis.* The eggs are resistant to drying and may widely contaminate clothing, bedding and house dust, thus maintaining a constant source of infection in homes and institutions. The parasite is cosmopolitan. It is especially common in children, probably largely because their insanitary habits allow greater chances of spread. Treatment of cases is useful in control, provided sanitary measures are taken to minimize reinfection from the local environment.

Related Species. Similar nematodes occur in many lower animals and are sometimes economically important as minor disease agents. Species in rodents are often easily obtained for study as are several related parasites of cockroaches.

Trichuris Trichiura. *Trichuris trichiura* is a frequent inhabitant of the cecum, appendix and upper colon of man. The basic morphology of *T. trichiura* is similar to that of *Trichinella spiralis,* page 810. The anterior three-fifths of the adult worm, containing the non-muscular esophagus, is attenuated. and the general appearance of the body has given rise to the common name "whipworm." Male and female worms are similar in size, 3 to 5 cm. long. The adult worms, embedded in the intestinal mucosa, have been accused of a role in the pathogenesis of appendicitis, but the evidence is inconclusive. In heavy infections they may cause intestinal disturbances and general toxic effects. Treatment with wild fig latex eliminates the worms, but only fresh preparations of the latex are effective. Enteric coated capsules of emetine have been reported useful in treatment.[93] The eggs passed in the feces are thick-walled with a plug at either end, 53 by 22 μ in size (Fig. 220). They are very resistant, surviving for months in contaminated soil. The embryos, undeveloped at the time of passage, require two weeks to several months, depending on temperature and humidity, to reach the infective stage. Ingested with contaminated food or water, they produce adult worms in the intestine. *Trichuris trichiura* infection is world-wide but especially abundant in the tropics and subtropics because of poor sanitation and the effect of climate on development of the eggs.

Ascaris Lumbricoides. Widely known simply as the "roundworm" of man, *Ascaris lumbricoides* is a large parasite of the small intestine. The white

[92] *Cf.* Rector: Amer. Jour. Trop. Med., 1943, 23:369.
[93] Burrows, Morehouse and Freed: Amer. Jour. Trop. Med., 1947, 27:327.

or flesh-colored females measure 20 cm. or more in length by 5 mm. in diameter, the males, 16 cm. by 3 mm. The characteristic eggs, 45 to 75 by 35 to 50 μ, have a smooth inner and a roughly tuberculated outer shell. They are undeveloped when passed in the feces (Fig. 220). In soil or water they become infective in a week or more, depending on environmental conditions. They may survive for several years despite drying, bacterial contamination or adverse chemical conditions. Ingested in food or water they hatch in the intestine. The embryos, however, do not develop directly to adults in this site. Gaining access to the circulation they are carried to the lungs and escape into the air spaces. Via the trachea they reach the pharynx and are swallowed. Partial development occurs during the above migration. It is completed in the small intestine, where egg-producing adults are found about two and one-half months after infection.

Passage of the migrating larvae through the lung may produce a severe bronchopneumonia. Larvae filtered out of the circulation in abnormal sites induce local inflammatory lesions which are serious in certain locations, such as the brain. Adult worms in the intestine often cause no symptoms. Particularly in heavy infections, however, intestinal and nervous disturbances may occur, and intestinal obstruction occasionally results. Various other injuries result from migration of the adult worms out of the normal habitat into the appendix, the bile ducts, the upper alimentary or respiratory tracts, the genitourinary system or through the intestinal wall into the abdominal cavity. Hypersensitivity develops in infected persons and in laboratory workers who have handled the worms, and severe anaphylactic reactions may occur in such individuals.

Immunity to reinfection is not known in man, but infection in animals with related parasites confers some temporary protection. Antibodies are detectable by various tests after either infection or vaccination with worm extracts, and considerable investigation has been devoted to antigenic analysis. An allergic skin test has been used in diagnosis but is often positive in uninfected individuals who have acquired a hypersensitivity from contact with the parasite. Laboratory diagnosis is preferably based on the finding of eggs in stools. However, a small proportion of cases harbor only male worms, and in such cases the immunological tests can be used.

Oil of chenopodium and hexylresorcinol are effective in removing the adult worms from the intestine. Some other drugs, particularly those used for treatment of hookworm infection (p. 807), may cause migration of the adult Ascaris into abnormal sites.

Man is the only known host of *A. lumbricoides*. Infection predominates in children; that this results in part from age resistance is suggested by the marked age resistance of animals to related parasites. Infection is world-wide but most prevalent in the tropics and subtropics, where sanitary and climatic conditions favor its dissemination. Control depends on the reduction of soil contamination by proper sewage disposal and treatment of infected individuals. Raw vegetables are an important source of infection and should be thoroughly washed.

Related Species. Cats, dogs, cattle, horses and other lower animals harbor a number of closely related species. Rare human infections have been reported

with at least three of these. A distinct race of *A. lumbricoides* causes a severe pneumonitis in young pigs, known as "thumps." In man the eggs hatch and the larvae reach the lung, but adult worms do not become established in the intestine. Similarly, the human *A. lumbricoides* develops only to the lung stage in pigs.[94] The adults of the pig Ascaris are easily obtained from slaughter houses for laboratory study.

Hookworms.[95] The two important hookworms of man, *Ancylostoma duodenale* and *Necator americanus*, will be discussed together. Known since antiquity, human hookworms were first accurately described by Dubini[96] in
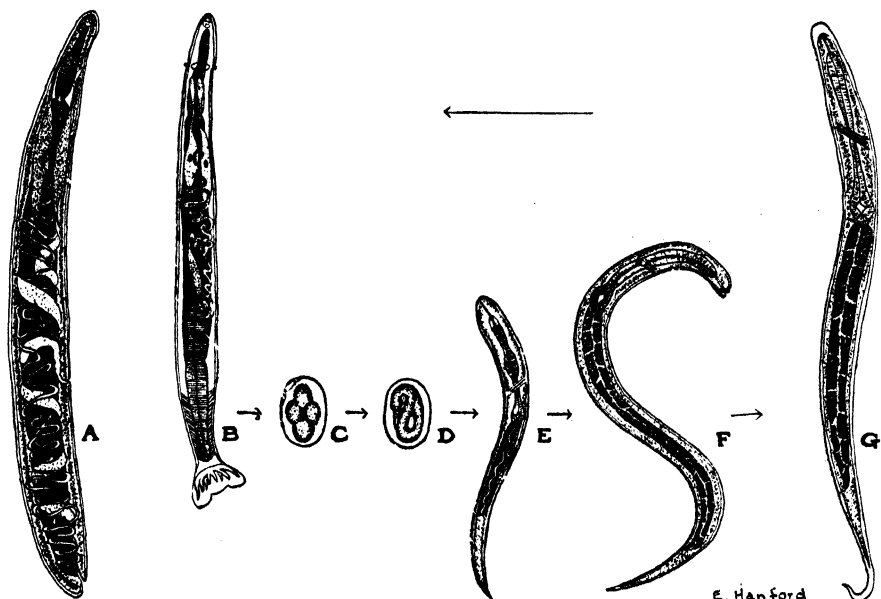
Fig. 223. Life-cycle of a hookworm, *Ancylostoma duodenale*. Note differences in magnification. *A*, Female worm. *B*, Male worm. *C* and *D*, Eggs. *E*, First stage larva. *F*, Second stage larva. *G*, Infective larva. *A* and *B*; × 10; *C–G*; × 250 (Adapted from Looss).

1843. Their relationship to disease was definitely shown by Perroncito[97] in 1880, and the life-cycle was elaborated by Looss.[98]

Characteristics and Life-Cycle. The adults of *A. duodenale* from the human small intestine are shown in Fig. 223. The female measures 1 to 1.5 cm. in length by about 0.5 mm. in diameter. The double reproductive system coils abundantly throughout the body cavity. Two pairs of prominent unicellular glands are seen in the anterior half of the body, one excretory in function, the other secreting histolytic enzymes. The dorsally directed mouth is characterized by two pairs of ventral teeth (replaced in *N. americanus* by curved cut-

[94] Koino: Japan Med. World, 1922, 2:317.
[95] For a comprehensive book see Chandler, *Hookworm Disease*. The Macmillan Company, New York, 1929.
[96] Dubini: Ann. Univ. di Med., Milano, 1843, *106*:5.
[97] Perroncito: Atti. Acad. d. Lincei, Roma, 1880, *7*:381.
[98] Looss: Ministry of Education, Egypt, Records of the School of Med., 1905–11, 3, 4.

ting plates). The male measures a little less than 1 cm. in length by about 0.4 mm. in diameter. The reproductive system shows a long, muscular ejaculatory duct, a prominent seminal vesicle and a coiled testis. At the posterior end of the male worm is a broad, flat clasping apparatus, the copulatory bursa, composed of cuticle supported by finger-like rays of tissue. *A. duodenale* and *N. americanus* can be identified by details of structure of this bursa as well as by the differences in buccal teeth noted above.

The thin-walled eggs measure about 38 by 58 μ. Those of the two species of human hookworms cannot be distinguished microscopically from one another. They are usually in early stages of development upon escape from the body, maturing and hatching within twenty-four hours if conditions are favorable in the soil. The larva, about 0.25 mm. in length, feeds on fecal material and grows in three days or more to a length of about 0.4 mm. After moulting it continues to grow until, by the fifth day or later, it measures about 0.6 mm. These two stages, characterized by a bulbous esophagus, are known as *rhabditiform larvae*. The third stage, resulting from a second moult, is a thinner form with a closed mouth and a less bulbous esophagus. It is usually still surrounded by a "sheath," the moulted skin of the preceding stage. This is the *filariform* larva, the infective stage for man. It does not feed but may survive long periods in the soil under favorable conditions. If the infective larva comes in contact with human skin, it actively penetrates through abrasions or hair follicles, reaches the circulation and is carried to the lungs. Here, like the migrating larva of Ascaris, it breaks out into the air spaces and reaches the intestine via the trachea and esophagus. After two further moults, the worms are mature. Egg production begins about one month after infection.

Hookworm Disease. Penetration of the skin by hookworm larvae produces a dermatitis known as "ground itch" or "miner's itch." The adult hookworms live attached to the mucosa of the small intestine, feeding on blood and bits of tissue. They move about frequently, leaving bleeding wounds in the intestinal mucosa. The chronic loss of blood causes anemia and edema, resulting, in severe cases, in retardation of growth and mental development and in weakness and general debilitation. Intestinal disturbances are seen in heavy infections. Death is rarely a direct result of hookworm disease, but the condition often contributes to death from other causes. In white persons the symptoms are directly related to the intensity of infection, which, because of the necessary development outside the body, is stable unless reexposure occurs. Individuals with fewer than twenty-five *N. americanus* virtually never show symptoms. Those with between twenty-five and one hundred worms show borderline effects. With more than one hundred worms some clinical injury is almost always detectable. *A. duodenale* is more harmful, about half as many worms being required to produce a given effect. Normally, infection is maintained by constant reexposure. In unexposed prisoners, however, it has been shown that about one half of the worms are lost in six months and about three quarters in two years. A smaller number persist for more than five years.

IMMUNITY. Puppies can be largely protected against the dog hookworm by repeated small infections.[99] Similar partial protection has been reported in a

[99] Otto and Kerr: Amer. Jour. Hyg., 1939, Sec. D 29:25; Otto: Jour. Parasitol. (Suppl.), 1938, 24:10.

human volunteer. That antibodies are involved is shown by the fact that some protection is conferred on puppies by immune serum.

DIAGNOSIS. Diagnosis of hookworm infection is based on recovery of characteristic eggs from the feces. Direct fecal smears were formerly used, but many infections with fewer than twenty-five worms are not detected with this method. Essentially all infections are revealed by the Willis flotation method, in which the eggs rise to the surface of a brine suspension of feces and are collected on a large microscope slide inverted over the container. Because the number of hookworms present in an infected individual is so important, several quantitative diagnostic methods have been devised. In the Stoll method a measured quantity (4 ml.) of feces is mixed with dilute NaOH solution (56 ml.) and all the eggs in a known sample volume (0.075 ml.) of this suspension are counted. The Lane method, or D.C.F. (direct centrifugal flotation), can be applied to lighter infections. One milliliter of feces washed by centrifugation is mixed with brine and recentrifuged with a cover slip on top of the tube. The eggs rise and can be counted on the cover slip as in the Willis method. Figures obtained by either of these methods are expressed as the number of eggs per ml. of feces. A single female *N. americanus* produces about forty-five eggs per ml. of feces (about 6000 eggs per day) so that twenty-five worms (males and females) produce about 600 eggs per ml. and 100 worms produce about 2500 eggs per ml. *A. duodenale* produces about twice as many eggs per worm, but because of the greater pathogenicity of this species the egg output gives a roughly equivalent index of the severity of infection in both species.

A number of drugs are effective in eliminating hookworms from the intestine. The best at present are tetrachlorethylene and hexylresorcinol.

Epidemiology and Control. Worms morphologically similar to *N. americanus* occur in pigs, but infection experiments indicate that they are distinct from the human hookworms. Man is the reservoir of infection with both *A. duodenale* and *N. americanus.*

There has been much study of the habits and requirements of hookworm larvae in the soil. The optimal temperatures are about 75° F. for *A. duodenale* and about 80° F. for *N. americanus.* Consistent high temperatures, about 100° F., are unfavorable. At 60° F. development is prolonged to two weeks or more, and below 50° F. there is little or no development. Continued temperatures below 40° F. kill the larvae. Moisture is essential to survival of the soil stages, but they die under water and are scattered by heavy rain. The type of soil is significant, probably largely in relation to its waterholding properties. Coarse sand and heavy clay are unfavorable, optimal development occurring in light sand or sandy loam. A large proportion of the infective larvae die within the first two weeks but a few survive for several months under favorable conditions. Although lateral migration from the site of development is insignificant, the filariform larvae can move considerable distances vertically in the soil. Unless excessive drying occurs, they remain at the surface, where the chance of contact with human skin is greatest.

In addition to the environmental requirements of the larvae, various human factors affect the distribution of hookworm infection. Fecal disposal practices and the use of shoes are among the most important. All these factors combine

to make hookworm infection a problem of the rural parts of warm countries. Exceptions occur in mines and tunnel-building operations, where local conditions may be favorable despite a generally unsuitable environment. *N. americanus* is the hookworm of central and southern Africa, the Western Hemisphere and southern India. Throughout the rest of southern Asia it is mixed with *A. duodenale,* and a few foci of *A. duodenale* occur in South America. In southern Europe, European mines and North Africa only *A. duodenale* is found.

In the southern United States hookworm infection is most common in children, largely because adults are protected from infection by wearing shoes. That age resistance, if it exists at all, is a minor factor is shown by the fact that in countries where the whole population goes without shoes infection may predominate in adults. Racial immunity is marked, and Negroes under comparable conditions have fewer and less intense infections than do whites. Furthermore, Negroes seldom show clinical symptoms even in the presence of heavy infections.

Control depends on the reduction of soil contamination by treatment of cases and proper fecal disposal and on the prevention of contact between human skin and the soil. Chemical destruction of larvae in the soil has been of use only in particular cases, such as mines. Education is the major weapon in hookworm control, for the chief problems are sociological, and biological knowledge of the parasite is adequate to insure successful hookworm eradication if the habits of infected populations can be sufficiently influenced. Treatment of cases has an important place in hookworm campaigns. Formerly it was customary to treat all infected members of a community, or even the whole community (mass treatment), if sample diagnoses showed abundant and heavy infections. Current opinion favors treatment of only the clinically significant cases. This has two great advantages. It simplifies the problem of preliminary surveys, since the search for infections may be limited to those population groups most likely to be heavily infected. In the second place, it avoids the educational disadvantages of treating persons who are not visibly benefited by the drug. In most regions the treatment of a small proportion of the population can eliminate most of the egg production.[100]

Hookworms of Lower Animals. Various animals harbor hookworms. Two species, *Ancylostoma braziliense* and *A. caninum* of dogs and cats, occasionally invade the skin of man. They are unable to develop further, and their wandering in the subcutaneous tissues produces a serpiginous dermatitis known as "creeping eruption." The adults of *A. braziliense* have been reported from man in the Far East. Other, more distantly related parasites are important disease agents of livestock.

Nippostrongylus muris of rats and mice is of interest because of extensive studies on the mechanisms of immunity. Serum antibodies have been shown to exert a direct action on the parasites *in vitro,* and these antibodies together with tissue cells immobilize and destroy the larvae in the skin and lungs and destroy adults in the intestine.[101]

Strongyloides Stercoralis. *Strongyloides stercoralis* is a common intes-

[100] Andrews: Amer. Jour. Pub. Health, 1942, *32*:282.
[101] Taliaferro and Sarles: Jour. Infect. Dis., 1938, *64*:35; *ibid.,* 1939, *64*:157.

tinal parasite of man in warm countries. It exhibits a life-cycle of extreme complexity, involving reproduction by adults in the soil as well as in the human intestine. Investigators working with different animal species of Strongyloides disagree on fundamental points and it is not clear whether or not these disagreements result from specific differences in the worms studied.

Only female worms are known in the intestine of man. They are thin, transparent worms 2 to 2.5 mm. long by about 0.05 mm. in diameter. They lie buried in the mucosa of the small intestine, and the thin-shelled eggs are deposited in the tissues, where they hatch to release rhabditiform larvae very similar to those of hookworms. Escaping in the feces the larvae undergo one of two types of development. They may moult to filariform larvae which pene-

Parasitic female --- -- -- --→ Eggs
in lung
↑
Parasitic female ←——— Adolescent female   Parasitic male
in intestine   in lung ←———┐   ┌- -- -- --→in lung
↓   │
Eggs   │
↓   │
Rhabditiform larvae   Larvae in circu-
in intestine --- ---┐   ┌--- --→lating blood
│   │   │   ↑
│   ↓   │
│   Filariform larvae
│   in colon
↓
Rhabditiform larvae ——————————→ Filariform larvae
in soil   in soil
↓   ↑
Free-living males and ——————→ Rhabditiform ——→ Free-living males
females in soil   larvae in soil   and females in soil

Schematic life-cycle of *Strongyloides stercoralis*. Broken arrows indicate rare or incompletely understood possibilities of development.

trate the skin to establish infection. Alternately, they may develop into the free-living males and females. The free-living female is stouter than the parasitic female and about 1 mm. long. The male is somewhat smaller, about 0.7 mm. in length. Eggs produced by the free-living female hatch to release rhabditiform larvae which may continue the free-living cycle or may develop into infective larvae. The infective larvae, whether produced by free-living adults or developed directly from the rhabditiform larvae in the feces, penetrate the skin of man and reach the intestine via the circulation and lungs as in the hookworms. Male worms have not been found in the intestine, but "parasitic" males like those in the soil have been reported[102] in the lung, and it has been suggested that fertilization occurs here or en route to the intestine, the males being quickly lost.[103] Since investigation of a related species in rats has shown that a single infective larva can give rise to a female in the intestine

[102] Kreis: Amer. Jour. Hyg., 1932, 16:450.
[103] Faust: *Ibid.*, 1933, 18:114.

producing fertile eggs, it is clear that the parasitic males are not essential in at least some species.[104] Oviposition in the lung has also been reported.[105] A final complication is introduced by the reports that rhabditiform larvae might transform into infective larvae in the colon and penetrate its wall or the perianal skin to produce hyperinfections.[106] The various paths of development indicated above are shown in the accompanying diagram, the dotted lines indicating phenomena which are incompletely known.

Penetration of the skin by the larvae causes dermatitis, and passage through the lung may give rise to a bronchopneumonia. The parasitic females cause intestinal disturbances characterized chiefly by diarrhea. Laboratory diagnosis is based on identification of the rhabditiform larvae in the feces. Gentian violet has been reported active against the female worms in the intestine, but there is disagreement concerning its efficiency.

The epidemiology of *Strongyloides stercoralis* infection is similar to that of hookworm, and the same sanitary control measures apply.

Trichinella Spiralis.[107] The "trichina" worm, *Trichinella spiralis,* was first seen in human muscles at necropsy by Tiedemann in 1821. Leuckart and others worked out the life-cycle.[108] The adult forms are intestinal parasites of man and many other mammals, but their sojourn in the intestine is so brief that the more persistent larval infection in the muscles receives the main emphasis. The infective stage for man is a larva in the muscles of the hog. This stage is a minute coiled worm, about 1 mm. in length, enclosed in a lemon-shaped fibrous cyst, 0.25 by 0.5 mm. in dimensions. Ingested by a susceptible host the cysts reach the stomach, where the muscle and cyst wall are digested away. In about two days the larvae mature in the small intestine to adult males and females. The male is a thin, transparent worm about 1.5 by 0.04 mm. (Fig. 224). The anterior half of the body is occupied by a non-muscular esophagus like that of the closely related *Trichuris trichiura.* At the posterior end are two pear-shaped clasping lobes. The female is about 4 by 0.06 mm. in size. After fertilization the females burrow into the mucosa where, after about one week, they begin to deposit minute larvae, about 0.1 mm. in length, in the tissues. These larvae reach the general circulation and are filtered out in the skeletal muscles. There they grow in about two weeks to the infective stage. One month after infection the larvae are surrounded by a fully developed cyst wall, produced by the host tissues. Much later, probably only after death of the larvae, these cysts become calcified, but they are known to remain infective at least for several months. Any skeletal muscle may be infected, but the larvae are most abundant in the diaphragm, intercostals, tongue, larynx and eye muscles. Meanwhile the adults have disappeared from the intestine. Most of the males die and are passed out within a week after infection. The females have largely disappeared within a month. It will be seen that the complete life-cycle occurs in a single individual, for infective larvae are

[104] Graham: Amer. Jour. Hyg., 1936, 24:71; 1938, 27:221; 1939, 30 (sec. D): 15; Jour. Parasit., 1938, 24:233; 1939, 25:365.

[105] Fülleborn: Arch. f. Schiffs.-u. Tropen.-Hyg. (Beih.), 1914, 5:26.

[106] Nishigori: Jour. Form. Med. Soc., 1928, 277:1.

[107] An excellent general discussion is: Gould: *Trichinosis.* Charles C Thomas, Springfield, Ill. 1945.

[108] Leuckart: *Untersuchungen über Trichina spiralis.* Leipzig and Heidelberg. 1866.

produced in the tissues of the same host which harbors the adults in its intestine.

Trichinosis. Trichinosis in man is characterized by two phases. Intense infection with the adult worms produces gastro-intestinal disturbances, usually with diarrhea. Occasionally the injury to the intestine is so severe as to result in death within a few days after infection. Muscle invasion by the larvae leads to varied signs of toxemia and muscle injury. Heavy infections cause death or chronic illness of long duration. The severity of the disease is proportional to the number of larvae ingested, and many mild cases undoubtedly escape detection.
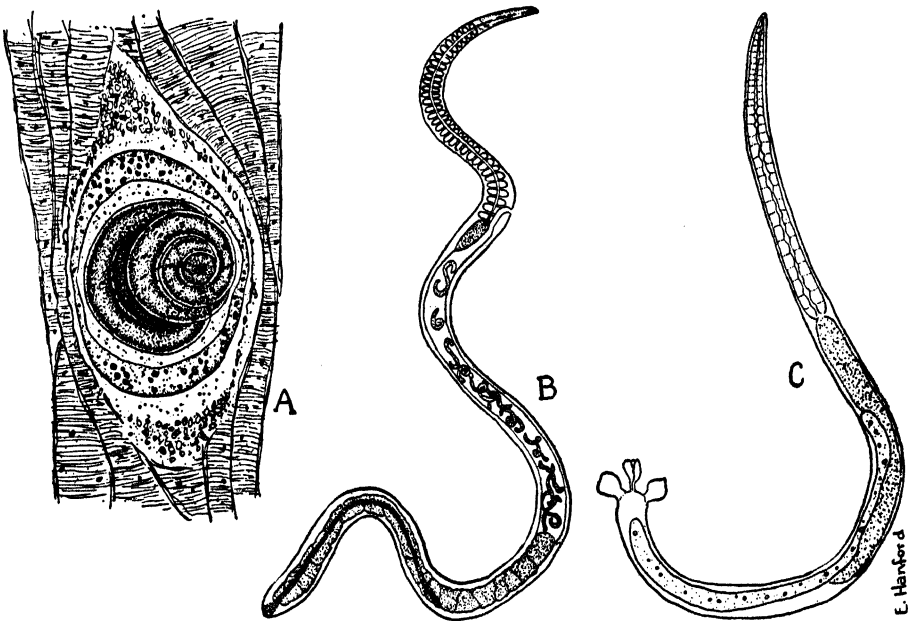
Fig. 224. *Trichinella spiralis. A,* Larva in muscle; × 100. *B,* Adult female; × 50. *C,* Adult male; × 50.

Immunity to superinfection is demonstrable in experimental animals and can be transferred with immune serum. Some protection may be produced by vaccination with killed larvae but this is insufficient to be of practical value.[109] Direct action of immune serum on the worms has been described. Precipitin and skin tests are available using extracts of larvae digested out of infected muscle.[110] These reactions are detectable two to three weeks after infection and remain positive for several years. Precipitating antigen has been detected in the serum as early as twenty-four hours after infection, and this phenomenon offers possibilities for early diagnosis.[111] Other diagnostic methods involve search for the larvae in body fluids or tissues. During migration they may be

[109] McCoy: Amer. Jour. Hyg., 1931, *14*:484; 1935, *21*:200.
[110] Bachman: Jour. Prev. Med., 1928, 2:513; 1929, 3:465.
[111] Bozicevich and Detre: Pub. Health Rep., 1940, *55*:683.

found occasionally in blood or spinal fluid, but it is often impossible to detect them. Muscle biopsies show the larvae in severe cases.

Specific drugs have not been available for trichinosis, but it has been reported that hetrazan reduces the numbers of adult and larval worms in experimentally infected rats.[112]

Trichinella spiralis occurs principally in man, hogs, rats and bears, although experimental infection has been produced in many species of mammals and birds. Human infection follows the consumption of undercooked pork, while pigs acquire the parasite from garbage containing pork scraps or, less commonly, by eating carcasses of infected rats. *Trichinella spiralis* is world-wide in distribution, though infection in the tropics is rare. In the United States routine necropsy examination of human diaphragms has revealed a general infection rate of between 14 and 28 per cent despite the fact that very few clinical cases are diagnosed.

Personal protection can be assured by thorough cooking of pork products, including hamburger of unknown composition. Government meat inspection does not attempt to detect Trichinella, for examination guaranteeing safety is considered impractical, but refrigeration for twenty-four hours at −18° C. destroys most of the larvae.[113] Laws which forbid the feeding of raw garbage to hogs have been successful in reducing the incidence of infection.

Wuchereria Bancrofti. Of the several species of "filarial" worms which parasitize man, *Wuchereria bancrofti* is by far the most important. The disease, elephantiasis, was known in antiquity, but the larvae were first seen in 1863 by Demarquay and the adults by Bancroft in 1876. Manson, in 1878, showed that development of the larvae took place in mosquitoes.[114] This discovery is noteworthy because it represents the first incrimination of an insect in the transmission of disease.

Characteristics and Life-Cycle. The adults live coiled in the lymph nodes of man. They are thread-like translucent worms, the female 7 to 10 cm. long by 0.25 mm. in diameter, the male 4 cm. by 0.1 mm. The egg, covered by a thin membrane, hatches in the uterus or in the host tissues to release an active embryo about 0.2 mm. long. This embryo, known as a *microfilaria*, escapes into the lymph and enters the circulating blood by way of the thoracic duct (Fig. 225). In most regions the embryos of *W. bancrofti* show marked nocturnal periodicity, occurring in the peripheral blood almost exclusively at night. In many of the South Pacific islands, however, they appear in the blood at all hours. Extensive study has failed to provide a satisfactory explanation of this periodicity.[115] There is no evidence as to the longevity of microfilariae in infected persons. At any rate, the microfilaria develops no further in the circulating blood. If ingested by a susceptible mosquito, however, it undergoes a series of transformations in the tissues of the insect. Within a day the larva leaves the stomach of the mosquito and invades the thoracic muscles. Here it develops into a stout "sausage larva," which, after a second moult, elongates to form the infective stage, a thin worm 1.5 to 2 mm. in length. This infective larva leaves

[112] Oliver-Gonzalez and Hewitt: Proc. Soc. Exp. Biol. & Med., 1947, 66:254.
[113] Augustine: Amer. Jour. Hyg., 1933, 17:697.
[114] *Cf.* Manson: Trans. Linn. Soc. London, 1884, 2:367.
[115] *Cf.* Hinman: Jour. Trop. Med. & Hyg., 1937, 40:200.

the muscles and migrates to the proboscis sheath of the mosquito, arriving six days or more after the infective blood meal. When the mosquito takes a new blood meal, the infective larva, apparently stimulated by the warmth of the skin, breaks out of the proboscis sheath and penetrates the skin, probably through the wound caused by the biting mosquito, to establish infection in man. The early development of the worms in man is not known, but micro-filariae appear in the circulating blood several months after infection. Although the longevity of the adult worms in the lymphatics has not been determined, they probably live for several years.

Filariasis. The adult worms are most prevalent in the lymph nodes of the inguinal region, but they are also found in lymphatics elsewhere in the body.

Fig. 225. Microfilaria of *Wuchereria bancrofti* in thick blood film; × about 150. (Courtesy National Institute of Health, U. S. P. H. S.)

They induce inflammation and fibrosis in the infected nodes. The consequent restriction of lymph flow causes edema, lymphangitis, lymphadenitis and elephantiasis. Secondary infection of the lymphatics with streptococci and staphylococci is often significant. Long-standing cases sometimes show enor-mous enlargement of the scrotum, vulva, legs or breasts.

Evidence of acquired immunity is lacking, but complement-fixing anti-bodies are detectable in the serum, and skin tests are positive in infected individuals. The reactions are group-specific, occurring with extracts of filarial worms from lower animals. Laboratory diagnosis is preferably based on the identification of microfilariae in fresh blood preparations or stained thick blood films. They are often not detectable in early infections or in long-standing cases with much tissue damage. Other filarial worms infecting man (see below) also produce microfilariae in the circulating blood, but these can be differen-

tiated microscopically from the larvae of *W. bancrofti.* Certain antimony compounds, notably neostibosan[116] and anthiomaline,[117] eliminate microfilariae from the blood of cases for long periods. Hetrazan has also shown promise in treatment. It is not known whether these drugs kill the adult worms in man, but they destroy the adults of other filarial worms in experimental animals.

Epidemiology and Control. Man is the only known host of the adult worm. A large number of mosquitoes of the genera Culex, Aedes, Mansonia and Anopheles support development of the larvae, but the most important are *Aedes variegatus* in the South Pacific islands, *Anopheles gambiae* in West Africa and *Culex quinquefasciatus* (*C. fatigans*) elsewhere. There is an interesting correlation between the habits of the mosquito hosts and the periodicity of the microfilariae discussed above. *Aedes variegatus* bites by day, and it is in the regions where this mosquito transmits *W. bancrofti* that the microfilariae are found in the peripheral blood at all hours. In other regions, where the principal vectors attack man in the evening or at night, the microfilariae show a marked nocturnal periodicity.

Because of a combination of factors, *W. bancrofti* is a parasite of warm countries, occurring widely throughout the tropics and subtropics. Climate favors the production of mosquitoes and the development of larvae of *W. bancrofti* in the insect, and the poorer housing of the tropics permits greater contact between mosquitoes and man. The slow development of human infections, the lack of multiplication of *W. bancrofti* in the mosquito hosts, and other hazards combine to limit dissemination of the parasite. As a consequence the infection seems to die out in an area unless there are many human cases and abundant mosquitoes. The only endemic center in the United States, around Charleston, South Carolina, where the incidence was 20 to 30 per cent before 1920, now shows very few cases. Control is a matter of mosquito reduction and protection from mosquito bites, attention being directed to the habits of the particular vectors in any area. Recent studies suggest that chemotherapy may be useful in control.

Other Species of Filarial Worms. *Wuchereria malayi,* a species very similar to *W. bancrofti,* occurs in southern Asia, often together with *W. bancrofti.* Various species of Mansonia and Anopheles are the vectors. *Loa loa* inhabits the subcutaneous tissues of man in Africa and is called the "eye worm" because of its habit of crossing the eyeball. Large temporary "calabar swellings" occur in the skin, but the infection is not serious. The microfilariae from the blood develop in deerflies of the genus Chrysops. *Acanthocheilonema perstans* and *Mansonella ozardi* are harmless parasites of human connective tissues. They are transmitted by midges of the genus Culiocoides. *Onchocerca volvulus* lives in nodules in the skin of man in Africa and Central America. The microfilariae do not occur in the blood but are found in the skin. In or near the eye they may cause blindness. Black flies of the genus Simulium serve as vectors.[119] Many other filarial worms are found in various mammals and birds.

[116] Culbertson *et al.:* Puerto Rico Jour. Pub. Health & Trop. Med., 1946, 22:139.
[117] Brown: Jour. Amer. Med. Assn., 1944, 125:952.
[118] Santiago-Stevenson *et al.:* Jour. Amer. Med. Assn., 1947, 135:708.
[119] *Cf.* Strong *et al.:* Amer. Jour. Trop. Med., 1938, 18, Suppl. to No. 1.

The dog heart-worm, *Dirofilaria immitis,* and *Litomosoides carinii* of cotton rats are much used in laboratory investigation.

The Guinea worm, *Dracunculus medinensis,* infects man and various lower animals. This parasite differs considerably from the other filarial worms infecting man. The small male worms are rarely seen. The females, after several months' development in internal connective tissues, appear in the subcutaneous tissues, usually of the leg. They are large worms, averaging about 1 meter in length, often visible through the skin as they lie in the tissues. The skin of the host ulcerates at the anterior end of the worm, and larvae escape, usually when the leg is submerged in water. These larvae develop in copepods, and human infection follows ingestion of the parasitized copepods in drinking water. The human infection, possibly the "fiery serpent" of the Bible, is found in Asia, Africa and parts of the West Indies and South America. In some regions the female worm is gradually removed from the tissues by rolling it up on a stick. Local injection of phenothiazine is said to be effective in therapy.

THE RICKETTSIAE[1]

The generic name Rickettsia is applied to a group of very small, gram-negative coccobacillary microorganisms which are the etiologic agents of the typhus fevers, spotted fever and related diseases, and tsutsugamushi disease of the Far East. They were first observed by Ricketts in 1909 associated with Rocky Mountain spotted fever. Those found in bodies of lice taken from typhus fever patients by da Rocha-Lima in 1916 were named by him *Rickettsia prowazeki* in honor of Ricketts, who had died in 1910 of typhus fever during an investiga-
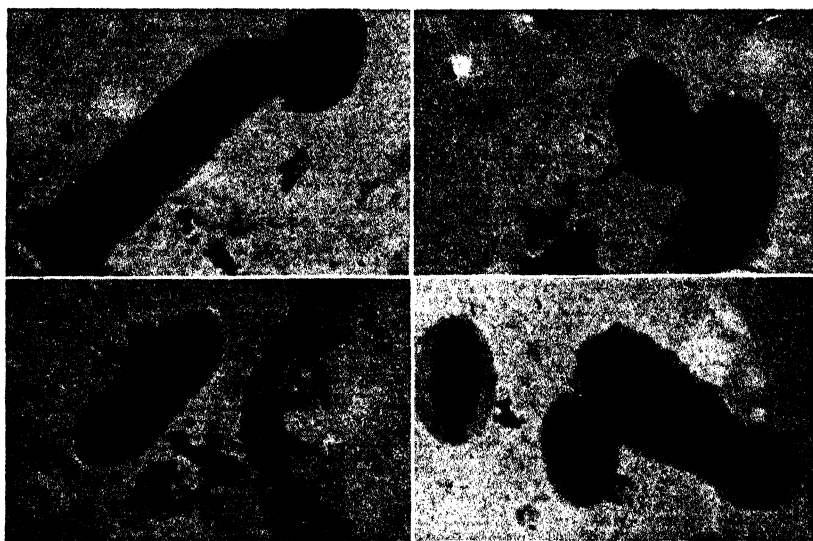
Fig. 226. Electron micrographs of *Rickettsia prowazeki* from egg yolk culture. Note the variability in size, ranging from coccobacillary to bacillary forms, and the capsular material surrounding the individual cells. (Lilly Research Laboratories.)

tion of that disease, and of von Prowazek, another early worker in the field who died of typhus.

Morphology and Staining. The rickettsiae are small coccobacillary forms and may appear either as cocci or as short bacilli (Fig. 226). They are usually 0.3 to 0.5 μ long and 0.3 μ wide, but bacillary forms may be as long as 2 μ. Examination with the electron microscope has indicated that they closely resemble the bacteria in internal structure, being homogeneous or

[1] For general reviews see Pinkerton: Bact. Rev., 1942, 6:37; National Inst. of Health Bull. No. 183, 1945; Topping and Shepard: Ann. Rev. Microbiol., 1947, 1:333.

slightly granular.[2] They occur singly, in pairs, and not infrequently in dense irregular masses in which no particular arrangement is apparent. Although sometimes found occurring free in the bodies of insects and infected persons and animals, the pathogenic forms proliferate only intracellularly and are usually observed, sometimes in large dense masses (Fig. 227), within the cells, particularly those of mesothelial origin which line the serous cavities. Some species of rickettsiae are found only in the cytoplasm while others invade the nucleus. They are non-motile and non-encapsulated. They are usually said to be non-filterable but *Rickettsia diaporica* readily passes diatomaceous earth filters.

The rickettsiae stain very poorly or not at all with the usual aniline dyes but readily stain a reddish purple with Giemsa. A buffered methylene-blue
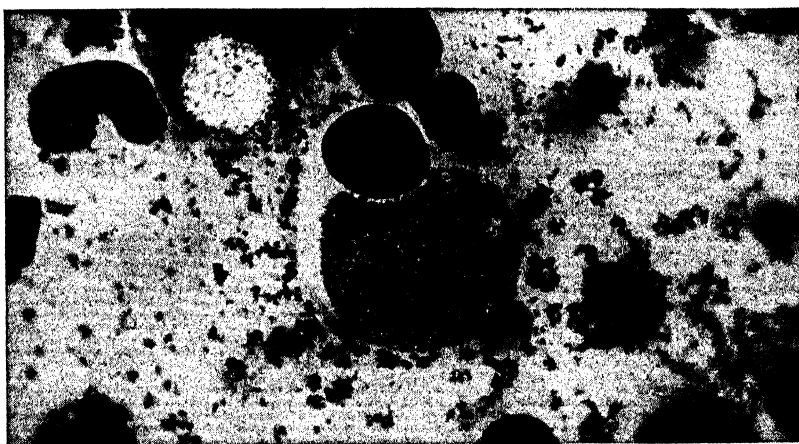
Fig. 227. Rickettsiae of Q fever in a smear of skin exudate. Note the macrophage packed with enormous numbers of the rickettsiae. × 1125.

stain and safranine counterstain devised by Castaneda stains rickettsiae a light blue. The coccus and coccobacillary forms stain evenly, but bipolar staining of the bacillary forms is not uncommon.

Cultivation. The rickettsiae resemble the filterable viruses in that, with a single exception, they have not been cultivated in the absence of living cells. The exception is *R. melophagi*, a non-pathogenic form found in the sheep tick *Melophagus ovinus*, which has been grown on glucose blood agar. Several of the pathogenic forms, however, have been grown in tissue culture, both in the usual plasma-tissue cultures and in various modifications of Maitland's tissue-Tyrode medium. They also develop on the chorioallantoic membrane of the chick embryo. The pathogenic rickettsiae so cultivated remain virulent, and reproduction of the disease with such cultures may be regarded as at least a partial fulfillment of Koch's second and third postulates.

The cultivation of rickettsiae has assumed particular significance as a means of producing large amounts of material for the preparation of vaccines. A number of methods have been devised for securing large numbers of rickettsiae, and,

[2] Plotz, Smadel, Anderson and Chambers: Jour. Exp. Med., 1943, 77:355.

although some of them are not cultivation methods, they may be conveniently considered here.

Use of Infected Insects. Infected insects have been used as a source of rickettsiae in the preparation of vaccines for spotted fever in this country and for classic typhus in Europe. Spencer and Parker[3] have prepared suspensions containing large numbers of spotted fever rickettsiae by grinding the viscera of infected ticks. The rickettsiae are killed with phenol. A similar method has been used by Weigl[4] for the preparation of vaccine material containing the rickettsiae of classic typhus fever. The viscera of lice infected by intrarectal inoculation are ground and the killed suspensions used as a vaccine. The yield is not great.

Use of Infected Animals. Heavy suspensions of the rickettsiae of murine typhus may be obtained from the peritoneal cavity of rats inoculated intra-
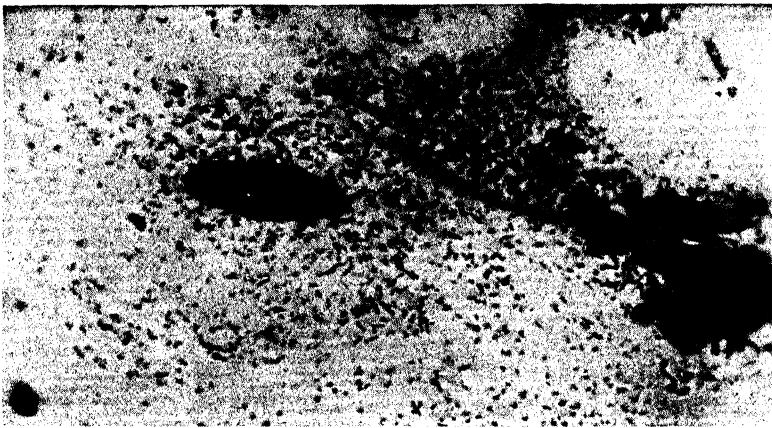
Fig. 228. Rickettsiae of Q fever in the yolk-sac emulsion from egg culture by Cox's method. The morphology is typical of rickettsiae in general; note the occurrence of paired microorganisms. × 1125.

peritoneally five days before. The resistance of the rats is reduced by x-ray irradiation, the injection of benzol or maintenance on a deficient diet. It has also been shown by Castaneda[5] that an experimental pneumonia may be produced in rats and other animals by the intranasal inoculation of murine typhus rickettsiae. The rickettsiae of classic typhus will produce a similar infection but very few are found in the lung tissue. Castaneda and Silva[6] have reported that multiplication of the classic type of typhus rickettsiae is much more pronounced when they are inoculated simultaneously with the murine variety. In either case enormous numbers of rickettsiae are found in the lungs and, when these are ground and centrifuged free from cellular débris, heavy suspensions of rickettsiae are obtained. Formolized suspensions prepared in this way are widely used in Mexico in prophylactic immunization against murine typhus fever.

[3] Spencer and Parker: Pub. Health Repts., 1925, 40:2159.
[4] Weigl: Bull. Internat. d. l'Acad. Polonaise d. Sc. e. d. Lettres, 1930, p. 25.
[5] Castaneda: Amer. Jour. Path., 1939, 15:467.
[6] Castaneda and Silva: Proc. Soc. Exp. Biol. Med., 1944, 57:80.

Culture. As indicated above, the rickettsiae proliferate only in the presence of living cells. Some type of tissue culture is, therefore, obligatory in their cultivation apart from the host. Although they may be grown on the chorioallantois of the developing chick embryo only small numbers are produced. They have also been cultivated in a modified Maitland medium (minced guinea pig tunica vaginalis suspended in a mixture of serum and Tyrode's solution). Zinsser, Fitzpatrick and Wei[7] devised a culture method which makes use of a modification of the Maitland method; minced embryonic chick tissue is inoculated with rickettsiae and spread on the surface of a Tyrode solution-serum agar slant. In these cultures proliferation of the rickettsiae is not associated with active host cell multiplication but rather with a low metabolic rate, and the rickettsiae appear to reach a height of proliferation as the host cells begin to die. Abundant multiplication has, in fact, been observed in dead chick embryos which contain surviving cells.[8]

A different and more successful method of growing rickettsiae has been devised by Cox[9] which consists of cultivation in the yolk sac of the developing hen's egg. The rickettsiae are found in huge numbers in the contents of the sac (see Fig. 228). It was believed for a time that extracellular multiplication occurred, but it is now known that proliferation of the rickettsiae occurs entirely within the lining cells of the yolk sac and they are liberated as the cells disintegrate. This egg culture method has been widely used for the preparation of vaccines.

Resistance. The rickettsiae are not hardy organisms and are killed by heating to 50° C. for a few minutes, and die out, usually within a few hours, at room or incubator temperature in infectious blood and similar materials. It has been reported, however, that sealed Maitland medium cultures remain infectious even up to a few months at 37° C. Rickettsiae may be preserved in infected tissues stored at —20° C. or in lyophilized material for several months. Like a number of the viruses they are resistant to glycerin, and tissues suspended in glycerin and stored in the refrigerator retain infectivity for several months. In general, however, they do not survive more than a few hours apart from the host cells. Their resistance to disinfectants is of the same order as that of the more delicate bacteria.

Pathogenicity. The rickettsiae appear to be well-established parasites of arthropods and there is good evidence for the acarinid origin of all the rickettsial diseases.[10] They are not pathogenic for the insect vectors which transmit them, except lice, and, in fact, the infection is hereditary in insects undergoing an incomplete metamorphosis, *viz.*, the ticks. They also appear to be reasonably well adapted to the animals, especially rodents, which in many instances constitute a reservoir of infection in nature. Disease may be produced experimentally in the guinea pig, however, the usual procedure in isolation of rickettsiae being the intraperitoneal inoculation of 5 ml. of blood in each of two animals.[11] A febrile attack occurs in seven to twelve days and some

[7] Zinsser, Fitzpatrick and Wei: Jour. Exp. Med., 1939, *69*:179.

[8] Rabinowitz, Aschner and Grossowicz: Proc. Soc. Exp. Biol. Med., 1948, *67*:469.

[9] Summarized in detail by Cox: Science, 1941, *94*:399.

[10] Huff: Quart. Rev. Biol., 1938, *13*:196.

[11] For a discussion of laboratory diagnostic procedures see Findlay: Proc. Roy. Soc. Med., 1941, *35*:157.

varieties of rickettsiae produce a characteristic orchitis or scrotal reaction. An acute fibrinous exudate is formed in the scrotal sac and many cells packed with rickettsiae may be found. The disease in the guinea pig is often not fatal, however, and this animal is much more resistant to infection than is man. Other laboratory animals are more resistant, and rats, mice, rabbits, dogs, cats, etc., may show no febrile reaction or other outward manifestation of disease though the infection has been established and in some cases, at least, persists for months.

The pathogenicity of the rickettsiae for man is often very high; certain strains of spotted fever rickettsiae produce a case fatality rate of 90 per cent or more and the rate in epidemic typhus may reach 70 per cent though it is ordinarily somewhat less than half that. Others, however, produce only a relatively mild disease. Variation in virulence from strain to strain for both experimental animals and man is marked. The various types of rickettsial infection in man are considered in later sections.

The characteristic pathologic changes found in rickettsial disease result from the multiplication of the microorganisms within the endothelial cells of the small blood vessels throughout the body, and especially in the skin and brain in the case of typhus fever. The inoculation of large doses of living typhus rickettsiae is toxic to experimental animals but the nature of the toxicity is not clear for killed rickettsiae are non-toxic; it has been suggested that possibly some metabolic product is toxic.

Immunity. Recovery from an attack of rickettsial disease usually confers a solid and lasting immunity. The development of artificial immunization procedures will be considered later but it may be noted here that the immune response is manifested by the appearance of protective antibody, of opsonins and agglutinins for the rickettsiae, and of complement-fixing antibody. The last has been of a good deal of interest as a differential diagnostic method and distinguishes even closely related varieties of rickettsia. Antibody neutralizing the toxicity of typhus rickettsiae occurs also and is used in the United States as the basis of assay of immunogenic potency of vaccines. The rickettsiae contain both somatic and capsular antigens. In the purification of yolk sac culture the material is extracted with ether to remove lipid material and, with the exception of *R. orientalis*, the rickettsiae are found in the aqueous phase. The extraction, however, results in the liberation of soluble antigen in the aqueous phase which has been found[12] to be stripped off capsular material.

The Weil-Felix Reaction. In some rickettsial diseases agglutinins appear to certain strains of Proteus. This apparently anomalous response was observed by Weil and Felix in 1915 and has very considerable diagnostic value. They found that a strain of *Proteus vulgaris*, which they designated X-2, was agglutinated by typhus sera, and another strain, designated X-19, was similarly agglutinated but to much higher titer; in fact, titers as high as 1:50,000 are occasionally observed in European typhus. It was further shown that the agglutinating antigen was a part of the heat-stable somatic or O antigen; these strains are, therefore, commonly termed OX strains. Other strains showing this immunological characteristic have been isolated since. It is of some importance that an O antigen be used in carrying out the agglutination because antibody

12 Shepard and Wyckoff: Pub. Health Repts., 1946, 64:761.

to the flagellar antigen of Proteus occurs with some frequency in normal sera. More general application of this agglutination test, commonly known as the Weil-Felix reaction, showed that it is specific for the typhus group of fevers, sera from other rickettsial diseases agglutinating only to low titer or not at all. As pointed out elsewhere (p. 430), the phenomenon is a consequence of the presence of a common antigen in *Rickettsia prowazeki* and the X strains of Proteus.

The Proteus agglutinin is only a part of the antibody response. It is not identical with the rickettsia agglutinin for the latter appears earlier, persists longer and appears to be associated with protective antibody. Furthermore, the opsonin remains in typhus serum after it has been absorbed with Proteus.

Another immunological type of Proteus, the OX-K strains, was found to be agglutinated by tsutsugamushi sera but not by typhus sera. This, coupled with

IMMUNOLOGICAL (WEIL-FELIX) GROUPING OF RICKETTSIAL DISEASES*

Immunological Group		
OX-19 OX-19 +++++ OX-2 + OX-K −	OX-K OX-19 − OX-2 − OX-K ++++	Undetermined OX-19 + OX-2 + OX-K +

Classic, European typhus
Brill's disease
Endemic, murine typhus of
  United   States,   Australia,
  Greece,   Syria,   Manchuria,
  Malay (shop typhus), India,
  Burma, Philippines, Hawaii,
  Toulon (fièvre nautique)
	Tsutsugamushi of Japan,
	  Formosa, Malay, Nether-
	  lands East Indies.
	"Scrub typhus" or "rural
	  typhus" of Malay, N. E. I.,
	  French Indo-China, India,
	  Australia
	Mite fever of Sumatra
		Spotted fever
		São Paulo typhus
		Fièvre boutonneuse
		Fièvre exanthematique
		Febbre errutiva
		Tick fever of South
		  Africa
		Kenya fever
		Tick typhus of India

* Modified from Felix: Brit. Med. Jour., 1942, ii:497.

the OX-19 type, makes possible the division of the rickettsial diseases into three groups, the typhus group in which OX-19 is agglutinated, the tsutsugamushi group in which OX-K is agglutinated, and the spotted fever group which is indeterminate in that neither type of Proteus is agglutinated to high titer. A classification of the rickettsial diseases on this basis is given in the accompanying table.

The significance of the Weil-Felix reaction is wholly unknown. The occurrence of one common antigen, or more specifically a polysaccharide haptene, in two such different microorganisms might of course occur purely by chance. The occurrence of a second common antigen, shared by another variety of Proteus and another group of rickettsiae, on the basis of chance alone is highly improbable. There is no evidence, however, which points to any other connection between Proteus and the rickettsiae. There is, for example, no association between the X strains and typhus; X strains are found in diseases other than typhus, and Proteus strains other than X strains are found in typhus. Proteus

	Rickettsia		Disease in Man
	Name	**Synonyms**	
Typhus group	*Rickettsia prowazeki* var. *prowazeki*...........	....	European typhus, classic typhus, epidemic typhus, louse-borne typhus Brill's disease
	Rickettsia prowazeki var. *mooseri*.............	*Rickettsia muricola*	Murine typhus, endemic typhus, tick typhus Shop typhus Toulon typhus (fièvre nautique) Moscow typhus Manchurian typhus Tabardillo (Mexican typhus)
Spotted fever group	*Rickettsia rickettsii*.....	*Dermacentroxenus rickettsii*	Rocky Mountain spotted fever, spotted fever
	Rickettsia rickettsii.....	*Rickettsia rickettsii* var. *brasiliensis*	São Paulo typhus Tobia fever (Colombia)
	Rickettsia rickettsii.....	*Rickettsia rickettsii* var. *conori* *Rickettsia conori*	Fièvre boutonneuse (Marseilles fever) Kenya typhus
	Rickettsia rickettsii.....	*Rickettsia rickettsii* var. *pijperi*	African tick fever
	Rickettsia akari........	....	Rickettsialpox
Tsutsugamushi group	*Rickettsia orientalis*.....	*Rickettsia tsutsuga-mushi* *Rickettsia nipponica* *Rickettsia akamushi*	Tsutsugamushi disease, Kedani fever, Japanese flood fever (Japan) Rural typhus, scrub typhus (Malaya) Mite fever (Sumatra)
Unrelated group	*Rickettsia diaporica*.....	*Rickettsia burneti*	Q fever American Q fever Nine-mile fever
	Rickettsia pediculi	*Rickettsia quintana* *Rickettsia wolhynica* *Rickettsia weigli*	Trench fever, Wolhynian fever

infections, of course, often result in the formation of specific agglutinins. It has been found, for example, that the sera of persons infected with *Pr. vulgaris*, *Pr. mirabilis* or *Pseudomonas pyocyanea* will agglutinate OX-K strains of Proteus to a high titer. Such persons might be said to give a positive Weil-Felix reaction.

Specific Serological Reactions. An accurate method of serological diagnosis and identification should, of course, be based on the use of rickettsial antigen and thus a true immunological specificity. Although it has been extremely useful, the Weil-Felix reaction is not a specific reaction and is not too reliable. Of the specific immunological methods, that of cross protection has been the most widely used until relatively recently, in large part because *in vitro* methods require antigen in quantities that were not available until culture methods were developed. While demonstrating immunological relationships, at least so far as protective antibody is concerned, the cross immunity test is not suffi-

Weil-Felix Type	Scrotal Reaction	Vectors	Vertebrate Reservoir	Geographical Distribution
OX-19	+	*Pediculus humanus*	Man	Europe, Asia, South America
OX-19	+++	*Xenopsylla cheopis* *Xenopsylla astia* *Polypax spinulosa*	Rat, squirrel, shrew	World-wide
Indeterminate	+++	*Dermacentor andersoni, D. variabilis, Hemaphysalis leporis-palustris* Possibly *Amblyomma americanum, A. cajennense, Dermacentor occidentalis, D. parumapertus, Ornithodorus parkeri, Rhipicephalus sanguineus*	Small rodents, rabbits, goats	Continental United States
Indeterminate	+++	*Amblyomma cajennense, A. striatum, A. brasiliense*	Dog, opossum	Brazil Colombia
Indeterminate	+++	*Rhipicephalus sanguineus* *Amblyomma hebraeum*	Dog Small rodents?	Mediterranean, South Africa
Indeterminate		*Amblyomma hebraeum, Rhipicephalus appendiculatus, Hemaphysalis leachi, Boophilus decoloratus*	Small rodents? Dog?	South Africa, Abyssinia
Indeterminate	+++	*Allodermanyssus sanguineus*	*Mus musculus*	Northeastern U. S.
OX-K	−	*Trombicula akamushi,* *Trombicula deliensis,* *Trombicula schuffneri* (Malay, Sumatra)	*Microtus montebelli* (Japan) *Mus concolor,* *Mus diardii* (Malay, Sumatra)	Japan Sumatra, Malay
Indeterminate	−	*Hemaphysalis humerosa* *Dermacentor andersoni, D. occidentalis, Amblyomma americanum*	Bandicoot Small rodents?	Australia, western United States
?	?	*Pediculus humanus*	Man?	Europe

ciently delicate to show differences in strains of *R. burneti,* for example, or between epidemic and murine typhus when the guinea pig is used as the experimental animal.

Cultures of rickettsiae in the mouse or rat lung and yolk sac cultures have been used as a source of antigen for *in vitro* serological reactions as well as immunizing antigens. In the first instance a suspension of ground tissue is differentially centrifuged to remove cellular débris, leaving a reasonably homogeneous suspension of rickettsiae which may be agglutinated by immune serum. Yolk sac cultures contain large amounts of lipid material which is removed by ether extraction of a saline suspension of the yolk sac material. As indicated above, all the rickettsiae except *R. orientalis,* which is found in the interphase emulsion, remain in the aqueous phage, and the treatment releases soluble antigen from all of these except *R. burneti.* The soluble antigen can be separated by spinning out the rickettsiae in the centrifuge and used in a

precipitin test, or the mixture of rickettsiae and soluble antigen can be used as an antigen in the complement-fixation test.

Difficulty has been experienced with some antigen preparations in that they tend to give false positive complement-fixation reactions, especially with Wassermann positive sera. This is not true of suspensions of washed rickettsiae but these are difficult and laborious to prepare. Van der Scheer, Bohnel and Cox[13] have, however, described the preparation of soluble antigens from infected yolk sacs purified by ether extraction, followed by treatment with benzene and precipitation with sodium sulfate. Complement fixation with this antigen does not give false positives with Wassermann positive sera, but does not always distinguish between epidemic and murine typhus.

Of the *in vitro* serological methods, complement fixation is by far the most satisfactory. It is usually carried out by the method outlined by Plotz[14] or that of Bengtson.[15] It has been extremely useful in the differentiation of closely related rickettsiae, in assaying the immune response to vaccine, and in the identification of rickettsiae and rickettsial infections.

Classification. The rickettsiae appear to be intermediate between the bacteria and the viruses. Most of the pathogenic bacteria that are obligate parasites in that they are incapable of a successful saprophytic existence are readily cultivable on artificial media, in some cases very simple media. In the host they may be actually outside the tissues proper as in the case of the dysentery bacilli and the cholera vibrio, but in most instances they are tissue parasites. With *Pasteurella tularensis* and *Bartonella bacilliformis* an intracellular existence is the rule but both can be cultivated on lifeless, though complex, media. The rickettsiae, while morphologically resembling bacteria, are intracellular parasites that cannot be grown in the absence of living cells and in this respect they resemble the viruses. In fact, the elementary bodies of certain of the larger viruses, such as psittacosis virus, are morphologically indistinguishable from small rickettsiae; conversely, rickettsiae found in the lungs of infected animals closely resemble the forms described in the developmental cycle of the psittacosis virus[16] (see also p. 879). The resemblance is also apparent in other respects for the pneumonitis produced by *Rickettsia burneti* very closely resembles that produced by the psittacosis virus. Clearly, then it is possible to set up a continuous series of parasitic types in which the ricksettiae occupy an intermediate position.

The question of the formal classification of the rickettsiae is another matter. As yet too little is known of the interrelationships of these microorganisms to permit subdivision on a basis other than that of expediency. Although it is clear that differences of generic status exist, it is generally agreed that formal classification is premature at present.

THE TYPHUS FEVERS

The typhus fevers constitute a group of closely related affections which occur in various parts of the world. The incubation period is five to eighteen days, and the clinical picture is essentially the same in all the typhus fevers, though

13 Van der Scheer, Bohnel and Cox: Jour. Immunol., 1947, 56:365.
14 Plotz: Science, 1943, 97:20.
15 Bengtson: Pub. Health Repts., 1944, 59:402.
16 Begg, Fulton and van den Ende: Jour. Path. Bact., 1944, 56:109.

the severity of the disease varies widely. There is an initial violent headache which persists with the onset of chills and high fever. A macular eruption appears soon after the fourth day which remains until defervescence. Crisis occurs at about the twelfth day, and recovery may be more or less complete at the end of another two weeks, though the cough which develops may persist and mental vigor may remain somewhat impaired for some time. The complications include typhus gangrene, which is perhaps associated with the circulatory disturbances arising during the disease, a highly fatal bronchopneumonia, otitis media and typhus encephalitis. Death rarely occurs before the end of the first week. The case fatality is highly variable; it has been as great as 70 per cent in some epidemics, and 20 to 30 per cent is not uncommon, while in endemic typhus it is much lower, perhaps 5 per cent. Typhus is almost always a considerably milder disease in children than in adults.

A single species of Rickettsia, *R. prowazeki*, is responsible for these diseases, and it characteristically occurs in the cytoplasm but not in the nucleus of invaded cells. There are two varieties of *R. prowazeki* which are associated with two epidemiologically different types of typhus fever. The one is the classic European or epidemic typhus and the variety of Rickettsia is termed *R. prowazeki prowazeki* by some workers. The other is murine typhus, sometimes called endemic typhus, and the variety of Rickettsia responsible is occasionally designated *R. prowazeki mooseri*. Both varieties are immunologically homogeneous and strains from all parts of the world appear to be substantially identical. The varieties may be distinguished from one another by complement fixation and evidently contain both common and individual antigens.

European Typhus Fever. The classic form of typhus fever known for many years is the louse-borne typhus of Central Europe which persists in endemic foci in Russia and Poland and has occasionally broken out in major epidemic form from time to time during periods of stress. The disease is associated with overcrowding and filth and has been termed "camp fever" and "jail fever." Epidemics are not infrequent in both civil and military populations during time of war and may become very extensive; it is estimated, for example, that 315,000 persons died of typhus in Serbia in 1915 and that 25 million cases occurred in Russia in 1917–1921.

The disease is transmitted by the human body louse, *Pediculus vestimenti.* The head louse may also transmit the infection, but its importance in the spread of the disease is not established. Not all lice become infected by feeding on typhus patients, but a considerable proportion, more than half, do. The lice become infective in five to eight days when kept at 32° C., and coincident with infectivity rickettsiae may be found in the gut. Although bedbugs and ticks have been experimentally infected, it is probable that the louse is the sole vector of the disease under natural conditions.

Brill's Disease. Louse-borne European typhus of a mild type is endemic, though in late years decreasing in prevalence, in cities along the Atlantic Coast in this country. Long regarded by many as different from European typhus, Brill's disease has been shown by Zinsser and Castaneda[17] and by Zinsser[18] on the basis of experimental and epidemiological evidence, to be louse-borne and

[17] Zinsser and Castaneda: New England Jour. Med., 1933, 209:815.
[18] Zinsser: Amer. Jour. Hyg., 1934, 20:513.

identical with European typhus. It has, in the opinion of these workers, been imported from Europe and become endemically established in cities with large immigrant populations.

The reservoir of infection in European typhus fever is not definitely known. The infection is not hereditarily transmitted in lice, and infected lice usually die within two weeks. It is supposed that the disease is maintained in endemic form by small numbers of mild infections. It is not known whether man can act as a healthy carrier of the infection.

Immunization. The preparation of vaccines which will produce an effective active immunity to typhus fever is a matter of some importance since under the circumstances in which it occurs in epidemic form adequate control of the vector is often not possible. A number of types of vaccine have been used, including the Weigl louse vaccine, living vaccines prepared from infected mouse brain, and suspensions of killed rickettsiae from rat lung or yolk sac culture.[19]

Of these the Weigl vaccine is impractical for use on any scale. Vaccine prepared from rat lung culture has been used to a considerable extent in Mexico and by the German army during World War II; the method of preparation of such vaccine, involving as it does intratracheal inoculation, is inherently highly dangerous and also relatively expensive. All of the rickettsiae may be cultured in the yolk sac of the fertile hen's egg and this has been the most extensively used method of growing rickettsiae. The vaccine currently used in the United States is yolk sac vaccine, prepared by ether extraction as noted above, and the final preparation is a suspension of formalin-killed epidemic typhus rickettsiae and soluble antigen. There is some reason to believe that the liberation of soluble antigen increases the immunogenic potency of the material. It is standardized on a toxicity basis, *i.e.,* prevention of the toxic effect of inoculated rickettsiae. It has been used in the United States Army, given as a course of three doses at seven- to ten-day intervals, followed by a single dose every four to six months. The possibility of allergic reactions following inoculation of the egg material has not proved to be important; approximately 6 to 8 million troops received vaccine with only two fatalities attributable to it.

Experimental evidence strongly suggests that a marked degree of immunity is produced by immunization. There is no good field test of its efficacy though only a very few cases of mild typhus and no deaths were recorded in the United States Army during World War II in spite of the exposure of many to the disease. There have been a number of instances of infection of vaccinated and unvaccinated laboratory personnel, and it is clearly apparent that immunization substantially modifies a subsequently acquired infection and the resulting disease is mild.

Murine Typhus. The type of typhus fever which prevails in the southern United States and in Mexico, where it is known as *tabardillo,* was long assumed to be a mild form of louse-borne European typhus. Maxcy[20] has shown, however, that the disease is associated with rats, and it has since become apparent through the investigations of a number of workers that the rat is a

---

[19] All of these vaccines and the general question of effective immunization have been discussed in some detail by Biraud: Bull. Health Organ, League of Nations, 1943, *10*:1.

[20] Maxcy: Pub. Health Repts., 1926, *41*:1213.

reservoir of infection in both this country and Mexico and that the disease is transmitted from rat to rat and from rat to man by the rat flea, *Xenopsylla cheopis,* and by the rat louse, *Polyplax spinulosa.*[21] The disease may also be transmitted by the human louse and, when a case occurs by transmission from the rat in a community where there are lice in abundance, murine typhus may become an epidemic louse-borne infection. Such epidemics occur from time to time in Mexico. Murine typhus occurs with considerable frequency in this country; approximately 20,000 cases were officially recorded in 1932–1941, and 5,191 cases with 214 deaths were reported in 1945 from 44 states. Georgia is the chief endemic center with about 1000 cases per year, the other southern states contributing the remainder. Isolated cases are reported from widely scattered regions including Boston, New York, St. Louis, Cleveland, etc., and the disease is apparently slowly spreading.[22] Control is essentially a matter of rat control.

Murine typhus also occurs elsewhere in the world and is known by various names. The urban type occurring in Malaya or "shop typhus," the mild form of the disease in the Mediterranean region known as *Toulon typhus,* and other infections such as *Moscow typhus, Manchurian typhus* and the *Red Fever of the Congo* are all murine typhus identical or nearly so with the type found in the United States and Mexico.

Clinically murine typhus does not differ appreciably from the classic European type. Both may exist in endemic and epidemic form and the differentiation between endemic (murine) and epidemic (European) typhus is not sound. It is not infrequently said that the European disease is the more fatal, but this does not appear to be true; both forms are equally fatal in epidemic form and relatively mild in the endemic form.

Differences between the two varieties of *R. prowazeki* are demonstrable but not great. The murine rickettsiae produce a necrotic scrotal reaction in guinea pigs, while the European variety does not; the murine variety may be carried indefinitely in mice without alteration, but the European variety tends to degenerate; the rickettsial pneumonia and the intraperitoneal multiplication in rats with the production of enormous numbers of rickettsiae noted earlier may be produced by the murine variety but not by the European type alone. There is a slight immunological difference also, for, while recovery from either infection results in a solid and lasting immunity to both, murine vaccines protect against murine infection but only incompletely against infection with the rickettsiae of European typhus. The two may be differentiated by the complement fixation test.

The Weil-Felix Reaction in the Typhus Fevers. As indicated earlier, the agglutination of Proteus OX-19 strains is a valuable adjunct in the diagnosis of these affections. The agglutinin appears relatively early, reaching a titer as high as 1:400 by the end of the first week and rising to 1:1600 or above by the end of the second week. It drops thereafter, falling to 1:400 or less by the eighth or ninth week after onset. There is, of course, great variation in the observed titers.

[21] *Cf.* Dyer *et al.*: Pub. Health Repts., 1931, 46:334, 470, 1869, 2481; Mooser, Castaneda and Zinsser: Jour. Exp. Med., 1931, 54:567.
[22] Topping and Dyer: Amer. Jour. Trop. Med., 1943, 23:37.

Normal serum from persons living in endemic localities often shows an agglutinin titer of 1:50 and titers as low as this are regarded as negative, 1:100 as suggestive, and 1:200 as diagnostic. The Weil-Felix reaction shows an anamnestic response in a number of febrile diseases, especially in typhoid fever. In typhoid fever, however, the titer of the Weil-Felix reaction does not continue to rise after the first week, whereas typhoid bacillus agglutinins continue to rise in titer.

Though classed as an indeterminate immunologic type, spotted fever frequently shows a considerable agglutinin titer for Proteus and differentiation
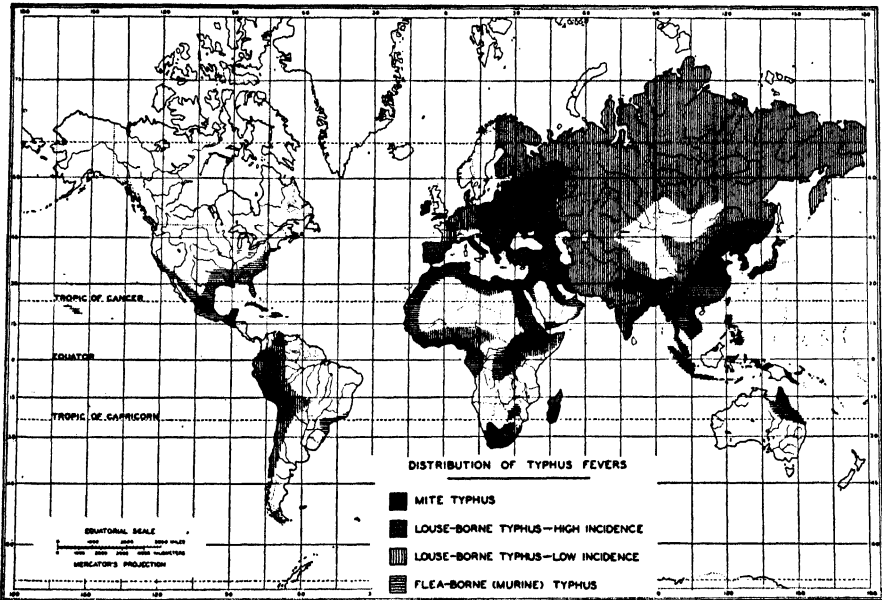
Fig. 229. The world-wide distribution of typhus fevers, including classic or European typhus, murine typhus and mite typhus of the Far East. Redrawn from maps prepared by Army Medical Intelligence, 1943. (Based on Goode *Base Map* No. 201M. By permission of the University of Chicago Press.)

from murine typhus may be difficult. Differential diagnosis may be established by complement fixation with rickettsial antigen.[23] Microscopic or macroscopic agglutination of rickettsial antigen appears to be practical but has not as yet been generally applied for diagnostic purposes.[24]

THE SPOTTED FEVERS

The rickettsial diseases which make up the spotted fever group are considerably more heterogeneous than those of the typhus fever group. Several of them are but poorly understood as yet. All are, however, transmitted by ticks, although some differ immunologically and clinically from Rocky Mountain spotted fever.

[23] Bengtson and Topping: Amer. Jour. Pub. Health, 1942, 32:48.
[24] *Cf.* Van Rooyen and Bearcroft: Edinburgh Med. Jour., 1943, 50:257.

Rocky Mountain Spotted Fever. Clinically Rocky Mountain spotted fever resembles typhus fever; the rash is generally more extensive and the nervous symptoms may be more pronounced, but in areas where both diseases prevail it is exceedingly difficult to distinguish them on clinical grounds alone.

The disease has been known for many years in the Rocky Mountain region in states such as Idaho, Montana, Wyoming, Oregon and Washington. In 1930, however, it was found in the eastern part of the United States in the South Atlantic states such as Maryland, Virginia, West Virginia and North Carolina. Although the disease is not limited to these regions, of the 2190 cases reported from 1933 to 1937, 65.5 per cent were from the Mountain and Pacific states and 27.4 per cent from the South Atlantic group; the two areas combined accounted for 93 per cent of the total cases reported in the country. In 1945 419 cases and 115 deaths were reported.

It is customary to speak of two types of the disease, an eastern type and a western type. Both are, however, immunologically identical and are caused by the same rickettsia, *Rickettsia rickettsii,* which, in contrast to the typhus rickettsia, is found within the nucleus of invaded cells. Both are transmitted by ticks, but by different species. The wood tick, *Dermacentor andersoni,* is the vector of the disease in the western states, and the dog tick, *Dermacentor variabilis,* transmits the disease in the Atlantic region. The rabbit tick, *Hemaphysalis leporispalustris,* disseminates the infection in the animal population. *Amblyomma americanum, A. cajennense, Dermacentor occidentalis, Rhipicephalus sanguineus, Dermacentor parumapterus* and *Ornithodorus parkeri* are able to transmit the disease experimentally and are to be regarded as potential vectors. The disease is hereditary, *i.e.,* is transmitted from one generation to the next, in *D. andersoni,* but whether it is hereditary in the dog tick is not clear. In the first instance, then, there need be no animal reservoir of infection. In the second, the dog may serve as a temporary reservoir of infection. The epidemiology of the eastern variety, however, is not well understood.

The case fatality of spotted fever is highly variable. In Idaho it has been about 4 per cent, in western Montana about 20 per cent, and in certain regions of the Bitterroot Valley it is as high as 90 per cent. Over the country as a whole, however, the rate is 18 to 19 per cent. It is generally said that the disease is milder in the Atlantic region than in the Mountain states, but over a period of years there is little difference in case fatality; for the period of 1933–37 the case fatality in the Mountain and Pacific states was 19.4 per cent and for the South Atlantic states 18.1 per cent.[25]

Spotted fever was transmitted to guinea pigs and monkeys by Ricketts in 1907. Guinea pigs show a febrile reaction, the spleen is enlarged, and the necrotic scrotal reaction occurs. *R. rickettsii* is immunologically related to the typhus rickettsiae but the titer of cross reaction in the complement-fixation test is not high enough to cause confusion; there is also a small degree of cross protection between the two.

A solid immunity appears upon recovery from the disease, and active immunization with the vaccine prepared from the viscera of infected ticks may be carried out. This vaccine confers full protection against mild strains but only occasional protection against the highly virulent strains of rickettsiae. The

[25] For further detail see Topping: Pub. Health Repts., 1941, 56:1699.

duration of the immunity probably does not exceed one year and annual re-vaccination is necessary. There is some evidence that persons who are suc-cessively vaccinated are better protected than those receiving but a single dose. Some fifteen years' experience with this vaccine has been summarized by Parker.[26] It has remarkable keeping qualities; three samples stored for twelve to fourteen years in the refrigerator were found to retain full potency.

The therapeutic use of immune serum has been a distinct possibility; recent experimental trial of immune rabbit serum in human cases of spotted fever has suggested that it has definite therapeutic value.[27]

São Paulo Typhus. The disease termed São Paulo typhus is not typhus but is immunologically identical with spotted fever. The symptoms are very similar to those of spotted fever, and the case fatality is very high, possibly 70 per cent. It also produces the necrotic scrotal lesions in the guinea pig. It is transmitted by the tick *Amblyomma cajennense*.

Fièvre boutonneuse (Marseilles fever) is a disease prevalent in the region of the Mediterranean and Black seas. The animal reservoir of infection is the dog, and the disease is transmitted by a hereditary tick vector, *Rhi-picephalus sanguineus*. The disease is immunologically very closely related to spotted fever but may be differentiated by complement fixation and differs somewhat in its clinical aspects. It runs a considerably milder course and the case fatality is only 1 to 2 per cent. There is also a primary sore and an adenitis of the regional lymph nodes.

Kenya fever is also immunologically identical with spotted fever and is transmitted by the tick *Rhipicephalus sanguineus*. It differs clinically, however, from fièvre boutonneuse in that there is no primary sore and no adenitis.

South African tick fever is a rickettsial disease, sometimes transmitted by *Rhipicephalus sanguineus* but more commonly by the larvae of *Amblyomma hebraeum*, a parasite of wild rodents. Clinically the disease is very similar to fièvre boutonneuse in that there is a primary sore and adenitis.

Q Fever. A rickettsial infection designated as Q fever has been described by Derrick and by Burnet and Freeman[28] in Australia which is immunologi-cally distinct from spotted fever although sera from patients do not agglutinate Proteus X strains. Infection in man has been found for the most part in abattoir workers. The bandicoot (*Isoodon torosus*) appears to harbor the dis-ease in nature and a number of other rodents and marsupials are susceptible to experimental infection.[29] It is transmitted among animals and possibly also to man by the tick *Hemaphysalis humerosa*. Guinea pigs and other laboratory animals may be infected experimentally. The scrotal reaction is not produced in the guinea pig. The rickettsia has been designated *Rickettsia burneti*.

The filter-passing rickettsia isolated by Davis in Montana produces a disease in guinea pigs which has been termed *nine-mile fever*.[30] The rickettsia has

[26] Parker: Amer. Jour. Trop. Med., 1941, *21*:369.

[27] Topping: Pub. Health Repts., 1943, *58*:757.

[28] Burnet and Freeman: Med. Jour. Australia, 1937, 2:299; *ibid.*, 1938, *1*:296; *ibid.*, 1938, 2:1114; *ibid.*, 1939, *1*:11.

[29] Derrick, Smith and Brows: Australian Jour. Exp. Biol. Med. Sci., 1940, *18*:409.

[30] Cf. the detailed studies of Parker: Pub. Health Repts., 1938, *53*:2267; Cox: *ibid.*, 1938, *53*:2270; Cox: *ibid.*, *54*:1822, 2171; Parker, Kohls, Cox and Davis: *ibid.*, 1939, *54*:1482; Davis: *ibid.*, 1939, *54*:2219.

been designated *Rickettsia diaporica.* Wood ticks (*D. andersoni*) transmit the infection under experimental conditions and the infection in the tick is hereditary; the animal reservoir of the disease in this country is unknown. It has been found that nine-mile fever and Q fever are immunologically very closely related, although the American strains are somewhat more virulent and the disease is now called Q fever regardless of where it is found. Naturally acquired infections in this country have been reported[31] and a number of laboratory infections have occurred[32] which substantiate the etiologic role of *R. burneti* in American Q fever.

With recognition of the disease it has become increasingly clear that Q fever is more prevalent in this country than had been supposed and, as in Australia, occurs in workers in meat packing plants.[33] It has been suggested that such infections are acquired from cattle ticks through the inhalation of dried infected feces. *R. burneti* has also been found in the Mediterranean area as a cause of atypical pneumonia among Allied troops in Italy during World War II and epidemics of Q fever have occurred among troops returning from Italy.[34] It is definitely indicated that sporadic and inapparent infections occur with some frequency in the Mediterranean area, and the etiologic agent of Balkan grippe has been found to be *R. burneti.*[35] The source of the human infection, insect vector and animal reservoir, if any, are as yet unknown.

In contrast to the typhus rickettsiae, *R. burneti* is not immunologically homogeneous and, though closely related, strain differences are apparent in the complement fixation test.

Whether South African tick fever and Q fever should be included in the spotted fever group is problematical. Their immunological dissimilarity separates them, but their clinical similarities and failure to agglutinate Proteus X strains suggest their inclusion in this group.

Bullis Fever (Lone Star Fever, Tick Fever). This disease was described by Woodland, McDowell and Richards[36] who observed it in soldiers stationed at Camp Bullis near San Antonio, Texas. About 1000 cases had been observed through 1943. The onset is abrupt, with fever lasting four to fourteen days and falling by lysis; lymphadenopathy and pronounced leucopenia are observed, together with a maculopapular rash on the trunk in severe cases. The case fatality rate is negligible.

Livesay and Pollard[37] established the infectious agent in guinea pigs by the intraperitoneal inoculation of patients' blood; a low-grade febrile reaction of forty-eight hours' duration was produced on the ninth to tenth day. Peritoneal scrapings and spleen smears showed rickettsia-like microorganisms, and similar

[31] Zemp: Jour. Amer. Med. Assn., 1943, *121*:838; Irons, Topping, Shepard and Cox: Pub. Health Repts., 1946, *61*:784.

[32] Dyer, Topping and Anderson: Pub. Health Repts., 1940, *55*:1945; Amer. Jour. Hyg., 1946, *44*:123; Huebner: Amer. Jour. Pub. Health, 1947, 37:431.

[33] Topping, Shepard and Irons: Jour. Amer. Med. Assn., 1947, *133*:813; Cox, Tesar and Irons: *ibid.,* 1947, *133*:820; Straus and Sulkin: Proc. Soc. Exp. Biol. Med., 1948, 67:139.

[34] Robbins *et al.*: Amer. Jour. Hyg., 1946, *44*:6, 23, 51, 64.

[35] Amer. Jour. Hyg., 1946, 44:110.

[36] Woodland, McDowell and Richards: Jour. Amer. Med. Assn., 1943, *122*:1156.

[37] Livesay and Pollard: Amer. Jour. Trop. Med., 1943, 23:475; *ibid.,* 1944, 24:281.

forms have been found in biopsy specimens from enlarged lymph nodes of patients. Convalescent sera fixed complement specifically with mouse spleen antigen and the disease appears to be immunologically distinct from Q fever and murine typhus.[37] The Weil-Felix reaction is negative.

It seems probable that the infection is transmitted by the tick, *Amblyomma americanum,* and there is some evidence that deer and rabbits may be infected. No name has been given to the rickettsia associated with the disease.

Rickettsialpox. A recent addition to this group of rickettsial diseases is rickettsialpox, a febrile disease characterized by an initial lesion and a vesico-papular eruption. It was observed in July, 1946, in New York City and 124 cases were reported. A rickettsia, named *Rickettsia akari,* was isolated from, patient's blood, and later from infected mites, which was immunologically related to *R. conori* by complement fixation but gave no Weil-Felix reaction and only incomplete cross protection against the Bitterroot strain of *R. rickettsii.* It is probable that the reservoir of infection is the mouse, *Mus musculus,* and the mite *Allodermanyssus sanguineus.*

TSUTSUGAMUSHI DISEASE (SCRUB TYPHUS, MITE TYPHUS)[39]

A disease of rickettsial etiology prevalent in the Far East is known as tsutsugamushi disease (dangerous bug disease), Japanese flood fever, or Kedani fever in Japan, as mite typhus in Sumatra, and as rural typhus or scrub typhus in Malaya. The last two are not to be confused with shop typhus of Malaya which is murine typhus. The disease is very similar to spotted fever in its distribution and clinical picture, but differs in the latter respect in that there is a primary sore and adenitis as in fièvre boutonneuse, and the symptoms include headache, orbital pain, a maculopapular rash and fever. As in spotted fever, the case fatality rate varies widely, from 0.5 per cent to perhaps as high as 60 per cent in some areas such as Korea. The disease is characterized immunologically by a Weil-Felix reaction in which the O antigen of the so-called X-K or OX-K strains of Proteus is agglutinated but the OX-19 strains are not.

The causative microorganism has been variously named *Rickettsia orientalis,* *Rickettsia nipponica, Rickettsia tsutsugamushi* and *Rickettsia akamushi;* the first, *R. orientalis,* is the name commonly used. Strains are closely related immunologically but are readily differentiated by complement fixation. There seems, however, no clear serological differentiation into types, and the strains also differ in virulence and in degree of adaptation to egg culture. The strains commonly used as antigens are the Gilliam and Karp strains, and sometimes the Seerengayee strain; sera from some cases are predominantly of one type or another, while others react to about the same titer with all three.[40] The Gilliam strain is toxic to mice while the others are not, and the toxicity is not neutralized by antisera to other strains of *R. orientalis,* or to only a slight degree.[41] *R. orientalis* may be grown in the yolk sac or chorioallantois of the developing hen's egg, in tissue culture and in the lungs of rats. Since this rickettsia occurs

[38] See Huebner, Stamps and Armstrong: Pub. Health Repts., 1946, *61:*1605.
[39] For a summary see Farner and Katsampes: U. S. Naval Med. Bull., 1944, 43:800; a summary of the subsequent work by the American Typhus Commission is given by Mackie, Davis and Fuller: Amer. Jour. Hyg., 1946, 43:195.
[40] Bengtson: Pub. Health Repts., 1946, *61:*887.
[41] Smadel, Jackson, Bennett and Rights: Proc. Soc. Exp. Biol. Med., 1946, 62:138.

in the emulsion phase in ether extraction of yolk sac culture, the material may be purified by extraction before saline dilution,[42] or by differential centrifugation[43] for the preparation of complement-fixing antigen.

Experimental animals recovered from non-fatal infection show practically complete cross immunity among strains of *R. orientalis.* Immunization with vaccine is a difficult matter, however, for apparently subcutaneous inoculation does not protect, but intraperitoneal vaccine protects against intraperitoneal challenge. Formalinized tissue culture vaccine or rat lung vaccine has been used for successful immunization of experimental animals.[44]

Tsutsugamushi occurs over a wide area in the Asiatic Pacific region, including Japan, Korea, Formosa, Indo-China, Malaya, Burma, Assam, New Guinea and the Philippine Islands. It appears to persist as an infection of rodents and of mites, and is transmitted to man by mites. The vertebrate host and insect host and vector differ somewhat from place to place. In Japan the rodent host is the field mouse, *Microtus montebelli,* and the disease is transmitted by the larvae of *Trombicula akamushi.* In Sumatra the vertebrate hosts are the house rat, *Mus concolor,* and the field rat, *Mus diardii,* and in Assam and Burma the wild rat, *Rattus flavipectus yunnanensis,* and the tree shrew,*Tupaia belangeri versurae.* Except in Japan the most important insect vectors appear to be *Trombicula deliensis,* a mite very closely related to *T. akamushi, Trombicula walchi* which is regarded by some as identical with *T. deliensis,* and *Trombicula fletcheri.* The mites occur in low, damp areas of grass, underbrush and scrub, in Japan along uncultivated banks of rivers, and the infection is thus restricted to certain localities. In the temperate climate of Japan the disease has a seasonal incidence, but in tropical regions there is no strict seasonal distribution of the disease in man.

TRENCH FEVER

In the course of the First World War a specific infection became known under the name of trench fever or Wolhynian fever. It is said to have caused almost one-third of all the illness in some of the armies in northern France and occurred also in Mesopotamia and Saloniki. It appeared again in the Second World War in the German Army in Russia.[45] It has a long incubation period (six to twenty-two days); the most constant symptom is pain in the legs; the fever is often high and of the relapsing type. Recovery is the rule. The disease can be transmitted to healthy men by the intravenous injection of whole blood taken from patients up to the fifty-first day of the disease.

Natural transmission is chiefly, if not solely, through the body louse. The bites of infective lice appear to be the main vector in producing trench fever, but the virus is also present in the excreta and may enter through abrasions in the skin caused by scratching. The virus is present in the urine of patients and is said to remain active for a long time in dry louse feces and dried urine.

The studies of Arkwright, Bacot and Duncan[46] established the fact that in

[42] Topping and Shepard: Pub. Health Repts., 1946, *61*:778.
[43] Bengtson: Pub. Health Repts., 1946, *61*:1403.
[44] Smadel, Rights and Jackson: Proc. Soc. Exp. Biol. & Med., 1946, *61*:308; Plotz, Bennett and Reagan: *ibid.,* 1946, *61*:313.
[45] Jacobi: Muench. Med. Woch., 1942, *89*:615.
[46] Arkwright, Bacot and Duncan: Jour. Hyg., 1919–20, *18*:76.

most cases lice or the excreta of lice that had been proved by inoculation to contain the virus contained large numbers of rickettsiae. Bacot, who became accidentally infected with trench fever, had for over two years previously been feeding upon his person a stock of lice known to be free from rickettsiae. Two days after the onset of his attack, he resumed the feeding of these lice, and rickettsiae were observed eight days afterward in enormous numbers in their excreta. Infection of the lice with rickettsiae continued possibly for as long as three months after the disappearance of all symptoms. Nothing, however, is known certainly about the occurrence of rickettsiae in the bodies of patients with trench fever. Further evidence is necessary to establish the causal relation of *Rickettsia pediculi* (also called *R. quintana*) to trench fever, although there is a strong presumption that such a connection obtains. The disease was not reproduced in experimental animals.

RICKETTSIAL DISEASE OF ANIMALS

In addition to the rickettsial diseases of man which often exist in nature in animal reservoirs of infection, other rickettsial infections occur in animals but not in man.

Heartwater Disease. This is a highly fatal disease of cattle, sheep and goats in South Africa. The causative organism, *Rickettsia ruminantium*, was described by Cowdry.[47] It is somewhat different morphologically from the typhus and spotted fever rickettsiae in that rod-shaped forms are rare, most of the organisms being rounded or elliptical and occasionally sharply curved. It has not been cultivated and laboratory animals do not appear to be susceptible to infection though succesful passage of the disease in ferrets has been reported.[48]

In infected animals the disease resembles the human rickettsia infections in that the vascular endothelium is parasitized, but differs in that large volumes of fluid accumulate in the pericardium, pleura and peritoneal cavity—hence the name heartwater. It is transmitted by *Amblyomma hebraeum*. Infection persists for some time after clinical recovery and immunity to reinfection is of a low order.

Other Animal Rickettsiae. Three other apparently closely related rickettsiae have been described in disease of animals.[49] *Rickettsia ovina* was found in sheep, *Rickettsia canis* in dogs and *Rickettsia bovis* in cattle. These rickettsiae are morphologically indistinguishable from one another and all are characterized by invasion of the circulating monocytes. They appear to be transmitted by ticks of the genera Rhipicephalus and Hyalomma.

[47] Cowdry: Jour. Exp. Med., 1925, 42:231, 253.
[48] Mason and Alexander: Jour. South African Vet. Med. Assn., 1940, 11:98.
[49] Donatien and Lestoquard: Arch. Inst. Pasteur (Algiers), 1937, 15:142.

Chapter 36

THE FILTERABLE VIRUSES

By F. B. Gordon, Ph.D., M.D.*

The existence of agents, apparently living but sufficiently small to be invisible under the microscope, and capable of passing filters which retain ordinary bacteria, was indicated by the work of Iwanowski in 1892[1] and of Beijerinck,[2] who found that bacteria-free filtrates of the juice of tobacco plants affected with tobacco mosaic would produce the disease in healthy plants. The similar filterability of the infectious agent of foot-and-mouth disease was soon demonstrated by Löffler and Frosch.[3] Since the beginning of the present century a great variety of infectious diseases of man, of lower animals and of plants have been shown to be due to similar agents, among them smallpox and the pock diseases of lower animals, rabies, poliomyelitis, yellow fever and a host of others. Reports of newly discovered filterable agents appear at frequent intervals and the list of so-called virus[4] diseases has steadily grown longer.

Following is a list of the more important virus diseases of mammals (including man) and fowls. The rickettsiae, closely related cytotropic agents, are covered in Chapter 35, and bacteriophage, which may be regarded as a virus disease of bacteria, is discussed in Chapter 38.

The pathogenic agents grouped under the head of filterable viruses defy precise definition, though in the aggregate they exhibit properties which are highly characteristic. As yet no descriptive statement sufficiently specific to have value and not requiring considerable qualification has been made concerning these agents. Though termed filterable, some viruses pass through bacteria-proof filters with difficulty or not at all, and although most viruses are not visible with ordinary microscopic technique—hence the term *ultramicroscopic virus*—some of the larger ones may be observed by special methods of illumination or in certain stained preparations. It is not infrequently stated that active immunity to a virus disease is usually solid, but in some instances immunity is quite transient. Ability to survive in 50 per cent glycerin is a property of many but not all viruses, and some bacteria also possess this property. Without exception, however, no virus has as yet been cultivated in the absence of living tissue cells, on even the richest of the artificial media

* Biological Department, Chemical Corps, Camp Detrick.
[1] *Cf.* Iwanowski: Centralbl. f. Bakt., II Abt., 1899, 5:250.
[2] Beijerinck: Centralbl. f. Bakt., II Abt., 1899, 5:27.
[3] Löffler and Frosch: Centralbl. f. Bakt., I Abt., 1898, 23:371.
[4] At present the term "virus," unless another meaning is obvious, is synonymous with "filterable virus."

suitable for cultivation of the most fastidious bacteria. There is also a lack of substantial evidence for an independent metabolism on the part of the viruses. Although all reports are not in complete agreement on this point, experiments[5] with purified vaccinia virus, to which special weight should be given, have yielded negative results. It may be noted, however, that some evidence of a respiratory mechanism has been detected in purified preparations of this virus (p. 850).

The failure to define these agents satisfactorily is a consequence of a lack of precise knowledge concerning them, for, although there is some information regarding their nature, many of them are, like the antigens and antibodies, known mostly in terms of what they do rather than what they are. It is quite possible also that viruses are a heterogeneous group of entities, taxonomically speaking, having in common only (1) size below a certain maximum (actually

VIRUS DISEASES[6]

Variola	Poliomyelitis
Vaccinia	Rabies
Influenza	Pock diseases of animals
Primary atypical pneumonia	Fibroma and myxoma of rabbits
Common cold	Papilloma of rabbits
Measles	Ectromelia of mice
Mumps	Pseudorabies (swine)
Herpes	Rinderpest (cattle plague)
Molluscum contagiosum	Foot-and-mouth disease (cattle)
Warts	Louping ill (sheep)
Psittacosis	Swine influenza
Ornithosis	Hog cholera
Lymphogranuloma venereum	Vesicular stomatitis (horse)
Trachoma	African horse sickness
Lymphocytic choriomeningitis	Equine infectious anemia
Yellow fever	Sarcomas of fowls
Dengue fever	Fowl plague
Phlebotomus fever	Fowl pox
Infectious hepatitis	Newcastle disease of fowls
Encephalitis (various types)	Infectious laryngotracheitis of fowls

the range of sizes is enormous, Fig. 236), and (2) habits of strict cellular, parasitism, regardless of how this arose.

In spite of the obvious and perhaps inevitable lack of exact definition of these agents, and present inability to differentiate them sharply from the bacteria in terms of any single characteristic, the filterable viruses in the aggregate attain a certain individuality which more than adequately justifies the unique position in which they are generally placed.

Cytotropism. The intimate and obligatory relation between the viruses and the cells of the host is the most important element in the definition of this group of agents, though it does not serve to set them off sharply from the rickettsiae; some species of bacteria and protozoa are also intimately associated with host cells.

Tissue Predilection. The viruses show, in most instances, a marked pre-

[5] Parker and Smythe: Jour. Exp. Med., 1937, 65:109.
[6] This list makes no pretense of being exhaustive and omits entirely the virus diseases of amphibians, fish, insects and plants.

dilection for certain tissues. A large group of viruses, including variola and vaccinia, molluscum contagiosum, trachoma and the pock diseases of lower animals, produce primary lesions of the epithelial surfaces of the body; viruses showing such affinities for the skin are termed *dermotropic.* Another large group, designated as *neurotropic* viruses, including rabies, poliomyelitis, encephalitis and others, produces lesions primarily in the central nervous system. Still other viruses, *e. g.,* virus of yellow fever, which attack the abdominal or thoracic viscera or produce signs indicative of generalized infection, are termed *viscerotropic.* The virus of influenza and certain others found in the lungs have been called *pneumotropic.* The term *pantropic* is used to indicate affinities for

Fig. 230. Foot-and-mouth disease. The section is from the tongue of a guinea pig and shows an early necrotic lesion of the epithelium. × 200 (obtained through the courtesy of Dr. I. A. Galloway).

many tissues; some prefer to reserve this term for viruses which are able to parasitize derivatives of all three primitive embryonic layers.

Such tissue affinities are not, however, absolute. The adjectives applied are indicative of the most apparent site of attack; secondary infection of other tissues not infrequently occurs, more commonly with some viruses than with others. As will appear, these tissue affinities may sometimes be altered by appropriate manipulation.

Effect upon the Host Cells. In general, the pathology of the virus disease runs the gamut of the possible types of tissue injury.[7] Necrotic and degenerative changes, acute and chronic inflammation and hyperplasia are all represented in varying combinations and locations. In most cases, it is generally believed, the effects are produced by actual invasion of the susceptible cells by the viruses, soluble toxins playing no part. A toxic action of the virus of lymphogranuloma venereum and others of this group (p. 875) has, however, been demonstrated. Heavy emulsions of this virus apparently act in the same

[7] *Cf.* Rivers: Amer. Jour. Path., 1928, 4:91.

manner as a bacterial endotoxin and antiserum will neutralize the effect of the toxin. Toxic effects of the virus of influenza (p. 867) have also been described.

Evidences of proliferation and degeneration are present in most virus infections, and the nature of the lesion is determined by the relative degree of each. Virus infections may be arranged in an orderly series according to the relative significance of these two factors.[8] At the one extreme are those viruses which produce degeneration and necrosis almost exclusively. Such, for example, are foot-and-mouth disease (Fig. 230) and the pock diseases. Severe necrosis of the liver cells is produced by the viruses of yellow fever (Fig. 252) and

Fig. 231. Rabbit papilloma. Hyperplasia of epithelial tissue is evident. × 125 (obtained through the courtesy of Dr. W. J. Purdy).

ectromelia. A greater amount of proliferation is seen in other instances, however; in fowlpox, for example, sometimes called epithelioma contagiosum, necrosis does not begin until after considerable hyperplasia has occurred. Viruses at the other extreme produce primarily a proliferation, and degenerative and necrotic changes play little or no part in determining the character of the lesion. Such is the case, for example, in rabbit fibroma, rabbit papilloma (Fig. 231), human warts and the filterable sarcomas of fowls.

In connection with the proliferative tendencies manifested in certain virus diseases, it may be noted that the filterable fowl sarcomas, of which a number have been described, have all the characteristics of malignant tumors.[9] They may be composed of very anaplastic cells, invade tissue rapidly, metastasize, and kill the host. The inoculation of susceptible fowls with cell-free filtrates of emulsified tumor tissue is followed by the appearance of a similar tumor, but similar transmission by cell-free filtrates of fowl tumors induced by tar

[8] *Cf.* Andrewes: Lancet, 1934, *ii*:63.
[9] *Cf.* the following reviews: Claude and Murphy: Physiol. Rev., 1933, *13*:246; Rous: *Virus Diseases*, Cornell University Press, Ithaca. 1943. pp. 147–170.

has been reported in only a few instances.[10] Rabbit papilloma, an infectious wart of cottontail rabbits, is due to a filterable agent. When transmitted to domestic rabbits these warts generally progress to true cancer and there is serological evidence that the papilloma virus persists in the malignant tumor, although it is not demonstrable by direct inoculation. It appears that the virus plays a significant, though not necessarily a primary, role in the production of the cancer.[11] Numerous attempts have been made to demonstrate filterable agents in malignant mammalian tumors, but the results have been almost uniformly negative.

Obvious evidences of tissue disturbance need not follow infection with filterable viruses, and *latent infections* with these agents occur just as they do with the pathogenic bacteria. With some viral infections, at least, *e.g.*, St. Louis encephalitis in the mouse brain, it appears that virus may increase to a fairly high titer before giving evidence of its presence. Beyond a certain level, however, histologic disturbance is recognizable and clinical signs appear. Healthy carriers of viruses exist among men and animals in the same way as carriers of bacterial or other infectious agents. Herpes virus (p. 862), well adapted to man, is carried by many normal individuals. A certain proportion of some stocks of white mice are healthy carriers of the virus of lymphocytic choriomeningitis (p. 885), and when the host-parasite equilibrium is disturbed by the intracerebral inoculation of sterile broth, an acute attack of disease ensues. Similarly, the virus known as Virus III may be latent in the testes of normal rabbits but produces an orchitis when testicular tissue is serially transferred, and a latent pneumotropic virus of mice (p. 883) is demonstrable by similar passage of lungs. Many of the plant viruses also produce such a latent infection; their presence may be demonstrated by passage to a susceptible strain of plant in which the manifestations of the disease will be evident. The practical significance of such latent virus infections to the study of experimental virus diseases is obvious.

Inclusion Bodies. The presence of the so-called inclusion bodies within the affected cell, either in the cytoplasm or in the nucleus, is in some cases another manifestation of the cytotropic character of the filterable viruses. These bodies are of varying size and appearance and are readily demonstrable in histological sections by differential staining. They are found in most, but apparently not all, virus diseases and are not found in bacterial infections.

Intracytoplasmic inclusion bodies are found associated with vaccinia (Fig. 232), molluscum contagiosum, variola, rabies (Figs. 233 and 257), fowlpox, infectious myxomatosis of rabbits, the viruses of the psittacosis-lymphogranuloma group (Fig. 249), trachoma, and certain others. They exhibit a considerable degree of individuality, and their recognition is of practical significance in the diagnosis of some virus diseases. One of the best known inclusion bodies in this connection is the Negri body (Fig. 233) found in rabies.

Intranuclear inclusion bodies are of two types. Type A, found in cells in the lesions of yellow fever, herpes (Fig. 243), varicella, B virus disease, Virus III of rabbits (Fig. 234), and others, consists of an acidophilic mass which

10 McIntosh and Selbie: Brit. Jour. Exp. Path., 1939, 20:49.

11 *Cf.* Rous, Kidd and Beard: Jour. Exp. Med., 1936, 64:385; Rous, Beard and Kidd: *ibid.*, 1936, 64:401; Kidd: *ibid.*, 1938, 68:703, 725, 737; Kidd: *ibid.*, 1940, 71:335, 351.

Fig. 232. Guarnieri bodies, the intracytoplasmic inclusion bodies of vaccinia. The arrows point to two inclusions among many in the infected corneal epithelium of a rabbit's eye. Giemsa stain; × 650.

Fig. 233. Negri bodies in human brain. This large nerve cell from the region of the hippocampus contained seven Negri bodies; five of various sizes are visible in the photograph. C = edge of cytoplasm of cell; N = edge of nucleus; NB = Negri bodies. Alzheimer's stain, reduced from × 2575.

disrupts the normal structure of the nucleus and may occupy a large fraction of the space within it. The accumulation of chromatin at the margin of the nucleus as seen in Fig. 234 is characteristic. Type B, described in Borna disease (an encephalomyelitis of horses), poliomyelitis, and a few others, consists of smaller bodies which characteristically are found within an otherwise normal-appearing nucleus. In contrast to the cytoplasmic inclusions, the nuclear inclusions do not have a morphology characteristic for the infection in which they are found. In fact, their significance in relation to virus infection is not at all clear, for they may be produced by other means as well. Inclusion bodies
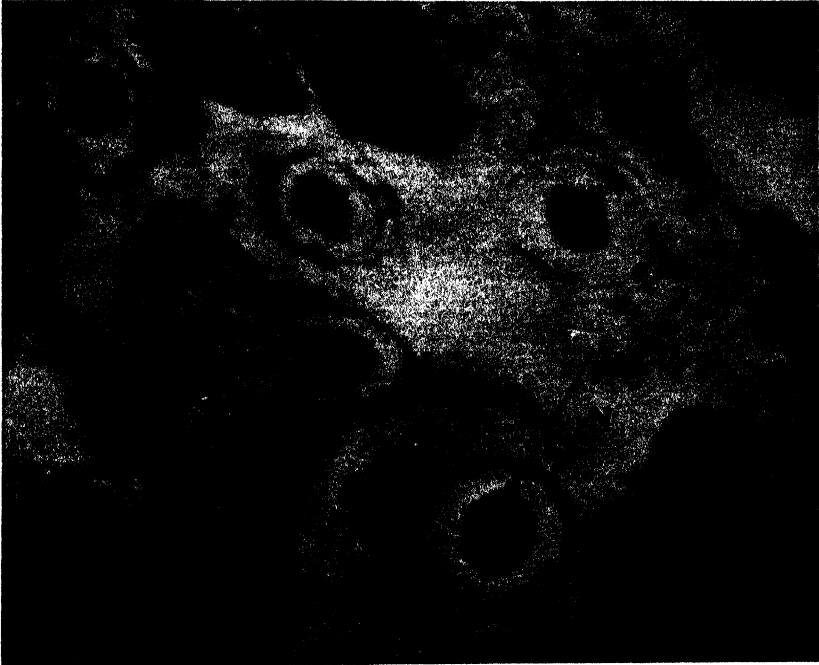
Fig. 234. Intranuclear (Type A) inclusion bodies of Virus III. I = inclusion body; N = marginated chromatin at the periphery of nucleus. This is from a section of infected rabbit testis. Giemsa stain; × 2550. (Obtained through the courtesy of C. H. Andrewes.)

resembling those of yellow fever, for example, have been found in the cells of the liver following severe burns. In other cases the inoculation of chemical agents, *e. g.*, the subcutaneous inoculation of guinea pigs with aluminum hydroxide, alundum or fresh or autoclaved brain tissue, has resulted in the appearance of nuclear inclusions in the macrophages.[12] The presence of intranuclear inclusion bodies, then, is not necessarily indicative of virus infection.

The Nature of Inclusion Bodies. Inclusion bodies have been regarded by some as products of cellular degeneration accumulated within the affected cell as, it may be noted, some of the nuclear inclusions not associated with virus infection may readily be. Some of the early workers suggested that these bodies were protozoan in nature and represented a stage in a complex life-cycle

[12] Olitsky and Harford: Proc. Soc. Exp. Biol. Med., 1938, 38:92.

of the microorganism. Von Prowazek[13] considered the inclusion body to be composed of a mass of small virus particles, which he called *elementary bodies,* surrounded by a mantle of amorphous material produced by the cell in reaction to the virus. On this basis he suggested that viruses should be called Chlamydozoa or "cloak animals." Since then it has been found that a number of intracytoplasmic inclusion bodies are essentially of this nature (Figs. 235 and 249). The evidence that the elementary body is the infectious particle of virus is presented below. It will suffice to state here that intracytoplasmic inclusion bodies, in some instances at least, may be regarded as "colonies" of virus within the cell.

The Physical Character of Viruses. Although Beijerinck postulated the presence of a *contagium vivum fluidum* in infectious plant juice, subsequent studies with animal viruses indicated that these agents are particulate
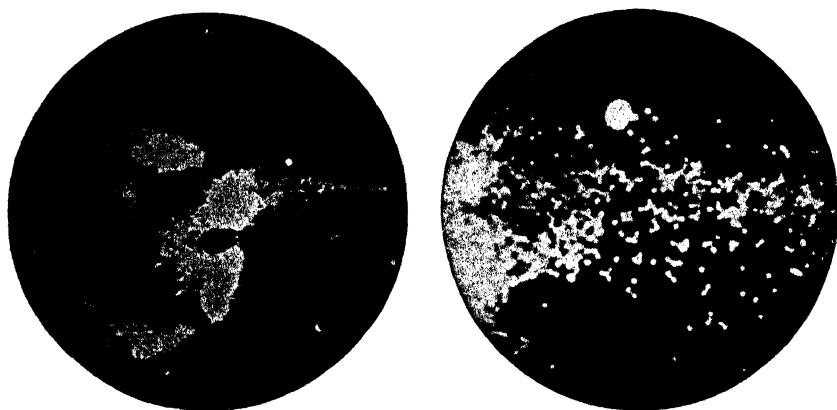
Fig. 235. Left, partially disrupted inclusion bodies of ectromelia from a culture on the chorioallantois of a duck egg. Darkfield illumination; × 600. Right, a portion of the same field under higher magnification showing the constituent elementary bodies. Darkfield illumination; × 2200. (From Himmelweit: Brit. Jour. Exp. Path., 1938, *19*:108. Reproduced through the courtesy of the author and publishers.)

in nature. Minute corpuscular elements, early noted by Borrel[14] in the lesions of fowlpox, were regarded by him as the causal agent of the disease. Von Prowazek and Paschen[15] described similar bodies in material from variola and vaccine lymph in which they were especially numerous. Similar elements have since been demonstrated in a number of virus infections and are known as elementary bodies (Fig. 242). Mention has already been made of their occurrence within inclusion bodies. Woodruff and Goodpasture[16] supplied the first direct evidence of the relation of inclusion body and elementary body to virus. They succeeded in freeing the inclusion bodies of fowlpox from other cellular material and demonstrated the infectivity of a single inclusion body. Furthermore, they showed that the inclusion body was composed of numerous

13 Von Prowazek: Arb. Gesundheitsamt, Berlin, 1905, 22:535.
14 Borrel: Compt. Rend. Soc. Biol., 1904, 57:642.
15 Paschen: Münch. med. Wchnschr., 1906, 49:2391.
16 *Cf.* Goodpasture: Harvey Lectures, 1929–30, p. 77.

	Diameter or width X length in mμ	
Red blood cells	7500	
B. prodigiosus (Serratia marcescens)	750	
Rickettsia	475	
Psittacosis	450	
Canary pox	260 x 310	
Myxoma	230 x 290	
Vaccinia	210 x 260	
Pleuro-pneumonia organism	150	
Pseudo rabies	150	
Herpes simplex	150	
Rabies fixe	125	
Influenza	115	
Newcastle disease	115	
Vesicular stomatitis	100	
Staphylococcus bacteriophage	100	
Fowl Plague	90	
T₂ coli bacteriophage	60 x 80	
Chicken tumor I	70	
Equine encephalomyelitis	50	
T₃ coli bacteriophage	45	
Rabbit papilloma (Shope)	44	
Pneumonia virus of mice	40	
Tobacco mosaic and strains	15 x 280	
Latent mosaic of potato	10 x 525	
Gene (Muller's est. of max. size)	20 x 125	
Southern bean mosaic	31	
Rift valley fever	30	
Tomato bushy stunt	26	
Poliomyelitis (Lansing)	25	
Hemocyanin molecule (Busycon)	22	
Yellow fever	22	
Hemocyanin molecule (Octopus)	20	
Louping ill	19	
Tobacco ring spot	19	
Japanese B encephalitis	18	
Alfalfa mosaic	17	
Tobacco necrosis	16	
Foot-and-mouth disease	10	
Silkworm jaundice	10	
Hemoglobin molecule (Horse)	3 x 15	
Egg albumin molecule	2.5 x 10	

Fig. 236. Approximate sizes of viruses and reference materials (Courtesy of W. M. Stanley, Chemical and Engineering News, 1947).

elementary bodies and that infectivity was associated with the latter. In the case of certain other viruses the presence of active virus in filtrates or centrifugates of infectious emulsions is directly correlated with the presence of elementary bodies. The size of the virus as determined by indirect means (see below) agrees well with the observed size of the elementary bodies. Furthermore, these bodies are agglutinated in specific immune serum. The intimate association between virus activity and the elementary bodies has become increasingly clear, and it is now certain that the elementary bodies are, in fact, the virus.

Size. The viruses show, as a group, an enormous range in size (Fig. 236). The smallest have a diameter, estimated by differential filtration, of approximately 10 mμ, and the largest have diameters as great as 250 mμ or more.

Filtration. As noted earlier, some viruses readily pass diatomaceous earth or unglazed porcelain filter candles, while others are filterable only with difficulty or not at all. Such filters do not, however, act as mechanical sieves, and it is well known that the electrical charge on the particles (like the bacteria, the viruses are negatively charged at neutrality) and that on the filter and, as a corollary, the nature of the suspending medium, all play a part in determining filterability.[17] The ability to pass through such filters is, therefore, related to particle size in only a crude way.

The use of graded collodion membranes or ultrafilters, frequently termed *gradocol membranes,* has added a quantitative element to filtration experiments, for these filters, though to some degree affected by charge, act much like mechanical sieves. Such membranes were originally introduced by Bechold[18] for the study of colloids. Elford[19] and others[20] have prepared membranes of known porosity[21] by drying solutions of collodion of varying concentration in thin sheets or by impregnating some carrier such as filter paper. The use of a series of such membranes of graded porosity allows a relatively accurate determination of particle size. Such filtration of viruses has been highly successful and much of our information concerning the size of viruses has been obtained in this way.

Ultracentrifugation. The size of virus particles also determines their rate of sedimentation in a centrifugal field. The high speeds required to throw down the minute virus particles necessitate special apparatus and the technique has been termed ultracentrifugation. Several methods have been employed. One, adapted from an earlier method of Schlesinger and modified by McIntosh and Selbie[22] and by Elford,[23] involves the titration of virus before and after centrifugation.

Another method of ultracentrifugation is an adaptation of that devised by Svedberg for the study of sedimentation rates of proteins. The high-speed

[17] Kramer: Jour. Inf. Dis., 1927, *40*:343.
[18] Bechold: Ztschr. f. phys. Chem., 1907, *60*:257; *ibid.,* 1908, *64*:32.
[19] Elford: Jour. Path. Bact., 1931, *34*:505; Proc. Roy. Soc., Ser. B, 1933, *112*:384.
[20] Allisbaugh and Hyde: Amer. Jour. Hyg., 1935, *21*:64; Bauer and Hughes: Jour. Gen. Physiol., 1934–35, *18*:143.
[21] The determination of porosity is based upon Poiseuille's law (*cf.* Cox and Hyde: Amer. Jour. Hyg., 1932, *16*:667) and is probably not strictly accurate.
[22] McIntosh and Selbie: Brit. Jour. Exp. Path., 1937, *18*:162.
[23] Elford: Brit. Jour. Exp. Path., 1936, *17*:399.

centrifuge is so arranged that ultraviolet light may be directed through the cell while the machine is in motion. Exposure of photographic plates at intervals allows sedimentation within the cell to be followed because of differential absorption of the light by various concentrations of sedimenting material. With homogeneous suspensions, *e.g.*, elementary bodies, a definite sedimenting boundary is visible (Fig. 237), and the rate at which the boundary moves represents the sedimentation rate of the virus particles. A relatively high degree of purification of the virus is usually a necessary preliminary to this type of observation.

An essential preliminary to the determination of particle size by sedimentation rate is knowledge of the density of the particle; other variables such as viscosity of the suspending medium, etc., can, of course, be controlled. Methods of estimating the density of virus particles are not entirely satisfactory, and there is considerable variation in the figures from different sources. The
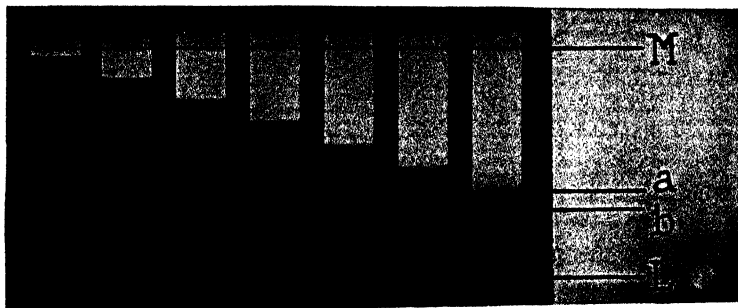
Fig. 237. Sedimentation of elementary bodies of vaccinia: *a* and *b* indicate the upper and lower edges respectively of the sedimenting boundary; the distance between constitutes the spread. From Pickels and Smadel: Jour. Exp. Med., 1938, 68:583. (Reproduced through the courtesy of the authors and publisher.)

density of vaccinia virus has been reported as 1.16, but this can be changed by inducing different degrees of hydration of the particle by employing suspending media of different osmotic pressures. There is evidence[24] that the density of some smaller viruses is nearer that of proteins, 1.30 to 1.35. A recent estimation of the density of influenza virus[25] gives 1.104. The values for particle size given for sedimentation rates approximate fairly well those obtained by filtration through gradocol membranes.

Microscopy. The stained elementary bodies of some of the larger viruses, including psittacosis (Fig. 247), vaccinia, fowlpox, canarypox, ectromelia and infectious myxoma, are resolved by the ordinary microscope with visible light. Silver-impregnation methods (Fig. 242), Victoria blue, or methylene blue and azur stains are suitable.[26] The size of the elementary bodies is, of course, distorted owing to the deposition of stain.

The larger elementary bodies may be seen in the ordinary darkfield microscope, though resolution is no better than with transmitted light. Incident

[24] Tang, Elford and Galloway: Brit. Jour. Exp. Path., 1937, *18*:269.
[25] Sharp, *et al.*: Science, 1944, *100*:151.
[26] *Cf.* Gildemeister, Haagen and Waldmann: *Handbuch der Viruskrankheiten*, Chap. 2. Gustave Fischer, Jena. 1939.

oblique illumination, giving an effect similar to that of a darkfield condenser, has also been used (Fig. 235), either alone or in conjunction with fluorescent dyes such as primulin.[27]

The resolving powers of the microscope are limited, as pointed out elsewhere (p. 41), by the wave length of visible light, and may therefore be extended through the use of shorter wave lengths such as ultraviolet. By this method, using quartz lenses, Barnard[28] was able to secure excellent photomicrographs of the elementary bodies of some of the larger viruses such as vaccinia, canarypox and ectromelia.

The electron microscope (p. 41) has been of great value in studying the size and shape of a number of viruses. Although there are difficulties in esti-
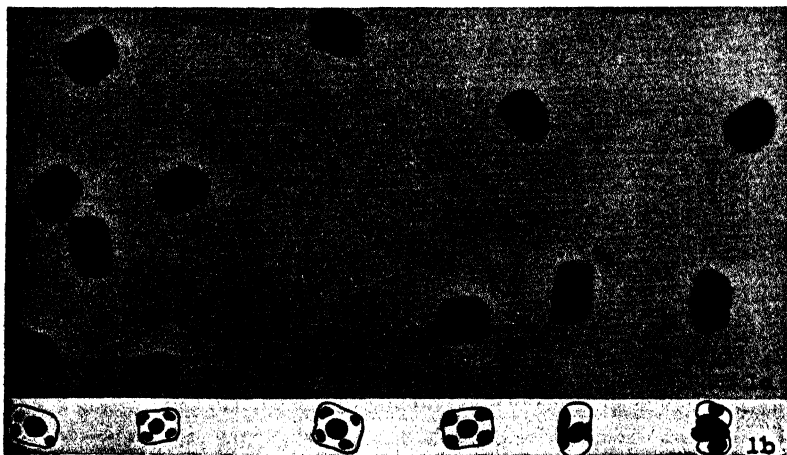
Fig. 238. *1a*, Structure of purified elementary bodies of vaccinia as revealed by the ordinary technique of electron microscopy. *1b*, Schematic representation of certain of the virus particles shown above. Magnification 7100×4 (From Green, Anderson and Smadel: Jour. Exp. Med., 1942, *75:*651. Reproduced through the courtesy of the authors and publishers).

mating size by this method, due to distortion during preparation, observations by this method agree well with determinations made by indirect methods. The technique of metallic "shadowing" is especially applicable to the study of viruses.[29] Electron micrographs are illustrated in Figures 238, 239, 240, and elsewhere.

Shape. A necessary assumption in the determination of particle size by filtration experiments is that of a spherical shape of the virus particle. That such an assumption was justified in many instances is indicated by the direct evidence of photomicrographs or electron micrographs. Nevertheless, examination of the "coccoid" elementary bodies of vaccinia by electron microscopy (Figs. 238 and 239) reveals that these bodies, when thoroughly washed, appear to be brick-shaped, or rod-shaped. Interesting evidence of internal structure is apparent in this virus and several others. Elementary bodies of the psittacosis

[27] *Cf.* Himmelweit: Lancet, 1937, *ii:*444.
[28] *Cf.* Barnard and Elford: Proc. Roy. Soc., Ser. B, 1931, *109:*360.
[29] Williams and Wyckoff: Jour. Appl. Phys., 1944, *15:*712.

group appear to possess an inner, more dense portion, surrounded by a less dense outer layer (Fig. 251). Certain other viruses have been shown to be cylindrical or filamentous in shape. Tobacco-mosaic virus consists of particles 15 by 280 mμ and there is evidence that other viruses may have a similar shape.

The Effect of Physical and Chemical Agents. Almost all experiments to determine the effect of physical and chemical agents upon viruses have necessarily been performed using impure virus preparations containing tissue elements and other extraneous organic matter. Although probably not giving correct values for pure virus, such experiments have obvious practical value. In some instances suspensions of elementary bodies or other purified preparations have given more reliable evidence concerning the resistance of the virus itself. For instance, tests with purified influenza virus[30] have confirmed in general the ideas gained in previous studies.
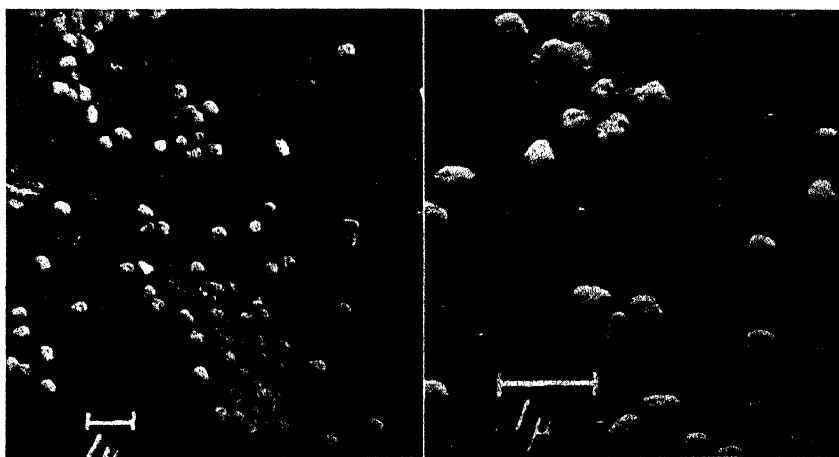
Fig. 239. Electron micrographs of shadow-cast preparations of the elementary bodies of vaccinia. (SAB No. 142.)

Heat. In fluid emulsions most viruses are inactivated by heat at about the same times and temperatures as vegetative bacterial cells; the rates of destruction of foot-and-mouth disease virus and *Salmonella typhi* are similar between 50° and 60° C. Some viruses appear to be somewhat more resistant, however. Glycerinated vaccine lymph is said to resist 55° C. for thirty minutes and hog-cholera virus in blood resists the same temperature for two hours. Vaccinia virus in the dried state is reported to resist 100° C. for five to ten minutes. Plant viruses vary considerably in their resistance to moist heat; thermal death points range from 42° to 90° C. for ten-minute exposures.

Extreme cold exerts a preservative action, and many viruses may be frozen, dried (lyophilized), and stored with only some initial loss of activity. Virus-containing tissues may be frozen and stored at temperatures obtainable with solid CO_2 (—76° C.) for long periods of time without deterioration, and in many laboratories a "dry ice" box is used routinely for storage of viruses.

Radiation. Direct sunlight inactivates viruses readily; presumably the ultra-

[30] Knight and Stanley: Jour. Exp. Med., 1944, 79:291.

violet portion of the spectrum is involved, for exposure to ultraviolet light results in a rapid inactivation. Fowlpox and vaccine virus are said to be more resistant than bacteria to ultraviolet light, the latter virus surviving in vaccine lymph when adventitious bacteria are killed. Although infectivity of a virus emulsion is easily destroyed by ultraviolet light, selection of the correct exposure time leaves the antigenicity of the preparation at a high level. This has been exploited experimentally in the preparation of several immunizing preparations.

Desiccation. Most viruses remain active for long periods in the dry state. Crusts from variolous patients are said to remain infective for years, and arti-
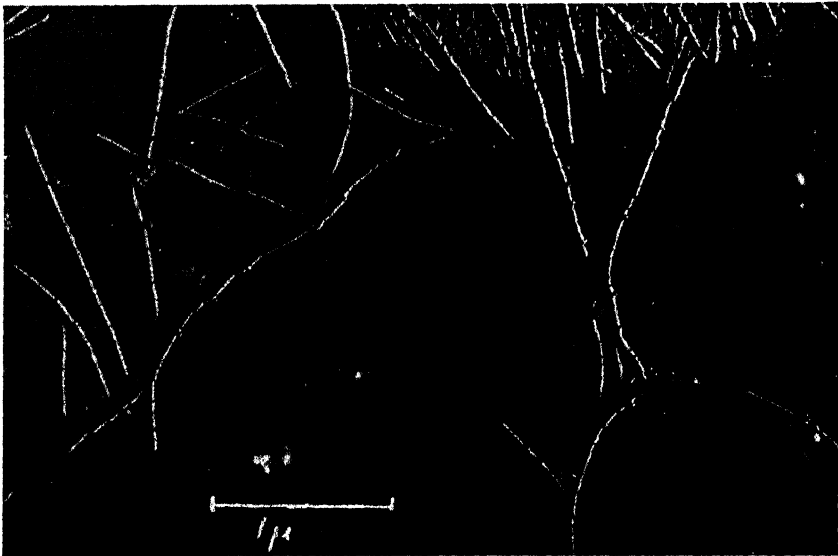
Fig. 240. Electron micrograph of a shadow-cast preparation of crystalline tobacco-mosaic virus protein. (SAB No. 135.)

ficially dried vaccine virus has been found active after four months at room temperature.

Chemical Agents. It is commonly stated that viruses are somewhat more resistant to the phenols than are bacteria, and this disinfectant is used to reduce the number of bacteria in vaccine lymph, leaving the virus active. Tests with vaccinia virus and others with purified influenza virus suggest that inactivation occurs readily with oxidizing agents (potassium permanganate, iodine), while foot-and-mouth-disease virus is resistant. Soaps and other detergents will inactivate influenza virus. Reducing agents, cysteine in particular, have been found to retard deterioration of virus preparations. A number of viruses are resistant to ether (vaccinia, poliomyelitis, rabies), but St. Louis encephalitis virus is quickly inactivated. Several viruses are inactivated by bile salts and urea, and many are rendered non-infective by treatment with formalin. Antigenicity is not destroyed by formalin, however, and immunizing preparations may be made in this manner.

The Chemical Composition of Viruses. Only a few viruses have been purified and prepared in sufficient quantity to allow a reasonably accurate chemical analysis. Some of the plant viruses have been the most thoroughly investigated because of the tremendous amount of virus activity present in the diseased tissues.

Tobacco-Mosaic Virus.[31] Pure proteins, intimately associated with virus activity, have been prepared from the juice of diseased tobacco plants by ammonium sulfate precipitation and by high-speed centrifugation. The protein appears to be the same irrespective of the species of the plant host, and forms needle-like "crystals" which are, in fact, paracrystals oriented in two dimensions only. These have more the properties of natural fibers and may be visualized as bundles of rodlike molecules lying parallel and overlapping one another in the direction of their long axis. The molecular weight of this substance is high; it has been estimated to be 40 million. Repeated recrystallization results in no loss of virus activity and, in general, inactivation coincides with destruction or alteration of the protein, which is now considered to be identical with the virus. Chemical analysis indicates that the substance is a nucleoprotein and a number of the amino acids present have been identified. It has been found that strain differences within the tobacco-mosaic virus group are reflected in quantitative and qualitative differences in the amino acid makeup of the molecule, with a preservation of the fundamental structural pattern.

Similar nucleoproteins have been found associated with the viruses of a number of plant diseases, and these likewise form paracrystals. The protein associated with the virus of bushy stunt of tomatoes, however, shows no evidence of asymmetry and forms true crystals.

Although all the animal viruses studied[32] so far have been found to have a high content of nucleoprotein they differ from the plant viruses in not being crystallizable and in possessing, in addition to the nucleoprotein, a lipid fraction, and in some cases a carbohydrate fraction apparently not accounted for by the nucleoprotein. Studies upon the dried elementary bodies of vaccinia,[33] a virus of relatively large size, have given values for protein, fat and carbohydrate not unlike those for bacteria. Desoxyribonucleic acid constitutes about 5.6 per cent of the elementary body and the figure for lipid is 5.7 per cent. Copper and a flavin-adenine-dinucleotide have been demonstrated to be integral parts of the elementary body, suggesting that an incomplete respiratory mechanism may be present. Biotin also is present in purified preparations of this virus.

The equine encephalomyelitis viruses contain a large amount of lipid, the figure being approximately 50 per cent, and in contrast to vaccinia, only the ribose type of nucleic acid is present. The influenza viruses contain 10 to 13 per cent of carbohydrate, an amount in excess of that contained in the nucleo-

---

[31] *Cf.* the following reviews: Stanley: Physiol. Rev., 1939, *19:*524; *Virus Diseases,* Cornell University Press, Ithaca. 1943, pp. 35–59; Wyckoff: Ergeb. d. Enzymforsch., 1939, 8:1; McFarlane: Biol. Rev., 1939, *14:*223.

[32] *Cf.* review by Beard: Jour. Immunol., 1948, 58:49.

[33] *Cf.* the review by Rivers: *Virus Diseases,* Cornell University Press, Ithaca. 1943, pp. 1–31.

protein, and both types of nucleic acid have been reported. The amino acid and carbohydrate analysis of strains A and B of influenza virus have been carried further.[34] Although both strains have similar amounts of some ten amino acids, as determined by microbiologic assay, significant differences in five others appear to reflect the strain difference.

Cultivation of Viruses. As noted above, the filterable viruses require the presence of living host cells for their proliferation; they cannot be cultivated on lifeless media. They are, like the spirochete of syphilis, often maintained in the laboratory by serial passage through susceptible animals. It may be noted that a number of viruses persist in infectious tissue for long periods of time when stored in the cold. *In vitro* culture methods have been devised, however, in which the virus is grown in the presence of living host cells either in tissue culture or in the developing hen's egg.
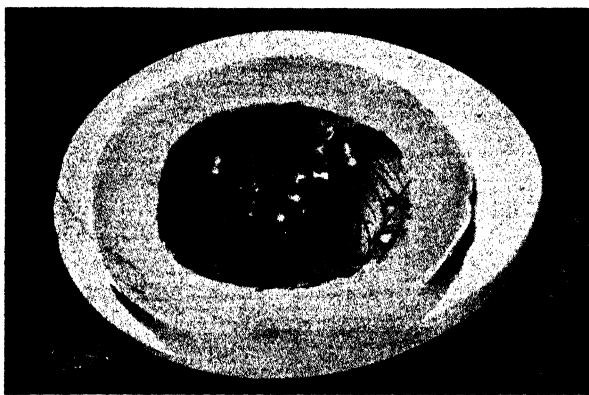
Fig. 241. Lesions produced by ectromelia virus on the chorioallantoic membrane of the developing hen's egg. (From Himmelweit: Brit. Jour. Exp. Path., 1938, *19*:108. Reproduced through the courtesy of the author and publishers.)

Tissue Culture. Many of the viruses may be propagated in tissue cultures such as those of Carrel in which there is proliferation of the tissue itself. Proliferation of the host cells is, however, apparently not essential, and viruses such as vaccinia have been cultivated by the Maitland technique in which fresh minced hen's kidney and hen's serum are incubated in a Carrel flask. Li and Rivers[35] have used minced chick embryo tissue in Tyrode's solution with or without serum. Under such circumstances there is little or no proliferation of the tissue cells, but their viability persists for some days. By this technique many viruses, including those of vaccinia, yellow fever, vesicular stomatitis, equine encephalomyelitis, St. Louis encephalitis, pseudorabies, influenza and others, have been cultivated. Other types of embryonic tissue may be used; the viruses of rabies and St. Louis encephalitis have been grown in association with mouse-embryo brain.

Egg Culture.[36] It was found by Woodruff and Goodpasture[37] that viruses

[34] Knight: Jour. Exp. Med., 1947, 85:99; 86:125.

[35] Li and Rivers: Jour. Exp. Med., 1930, 52:465.

[36] *Cf.* monograph by Beveridge and Burnet: Med. Res. Council (Great Britain), Spec. Rept. Ser. 256, 1946.

[37] Woodruff and Goodpasture: Amer. Jour. Path., 1931, 7:209.

may be cultivated on the chorioallantoic membrane of the developing chick embryo. Some gross alteration of the membrane is usually produced, and when the dilution of inoculated virus is suitable, isolated opaque lesions (consisting of hyperplasia, necrosis and inflammation) called "pocks" may be found (Fig. 241). Some viruses, when placed upon the chorioallantoic membrane, invade the body of the embryo; louping-ill virus may be found in the brain and liver, for instance, and St. Louis encephalitis virus invades the chick brain. With repeated passages many viruses gain in virulence and kill the embryo. Influenza virus and others will infect the respiratory tract of the embryo when inoculated into the amniotic cavity. An important advance in the study of influenza virus was made when it was discovered that a good growth of the virus occurs after inoculation into the allantoic cavity.[38] The virus is recoverable from allantoic fluid in a relatively pure state.

The yolk sac of the fertile egg was found by Cox to be especially suitable for cultivation of rickettsiae (p. 819) and the technique has proved to be excellent for growing viruses of the psittacosis-lymphogranuloma group (p. 875).

Variation.[39] Adaptive response to environmental conditions, a reaction associated with living organisms, is exhibited by many, perhaps all, viruses. Although observable characters are few, alterations in the virulence, or pathogenicity, and immunological character of the viruses are well known.

As in the case of the bacteria, strains of a given virus are found in nature which differ from one another in virulence and antigenic character. The so-called benign and malignant varieties of variola (p. 859) differ considerably in pathogenicity, as evidenced by case fatality rates. Similarly, the eastern and western varieties of equine encephalomyelitis differ, the former being the more virulent. These two varieties show immunological differences, and strains of other viruses, such as those of influenza A and others, are also immunologically different in minor respects.

Adaptation. Alterations in virulence and other characters may occur under the controlled conditions of the laboratory. One of the best known examples is seen in the conversion of rabies "street virus" to "fixed virus." This is accomplished by serial intracerebral passage in the rabbit and results in a gradually increasing virulence as evidenced by shortened incubation periods until the limit is reached. The virulence then remains constant at that level. During this process the strain loses certain characteristics including its ability to infect the central nervous system after subcutaneous inoculation, and its ability to produce Negri bodies.

Adaptation to a laboratory species with loss of virulence for the natural host has been observed repeatedly, and exploitation of such adapted strains for active immunization has been effective in some instances, *e.g.*, yellow fever virus, page 891. Alterations in pathogenicity as expressed in changed tissue affinities are frequently observed. Passage of the "normal" (dermotropic) strain of vaccinia through rabbit testis and then to rabbit brain has given rise to a neurotropic strain of enhanced virulence which produces an encephalitis in rabbits. Yellow fever virus, in nature both neurotropic and viscerotropic, has been observed to lose its viscerotropism during serial passage in mouse brain. Cultivation may produce similar changes in some instances; a neutropic strain of the

[38] Burnet: Austral. Jour. Exp. Biol. and Med. Sci., 1941, *19*:291.
[39] *Cf.* the review by Findlay: Jour. Roy. Microscop. Soc., 1936, *56*:213.

influenza virus has been produced[40] by cultivation of the naturally occurring pneumotropic form in the chick embryo, producing first a hemorrhagic encephalitis of the embryo and, on serial passage of the culture virus through mouse brain, an encephalitis in mice.

"*Mutation.*" The changes described above are perhaps to be regarded as gradual adaptive responses to the environment, possibly analogous to similar changes in bacteria and the *Dauermodifikationen* of higher forms. Sudden alterations, termed by some "mutations," are also observed. Such, for example, is the sudden, apparently spontaneous, variation of the rabbit fibroma virus to give, not the tumor-like proliferative fibrous lesion, but a lesion characterized chiefly by inflammation.[41] An induced conversion, superficially analogous to the transmutation of pneumococcus types (p. 182), of rabbit fibroma virus to the immunologically related virus of infectious myxoma has been reported.[42]

Reversion. The alterations in a virus arising, presumably, as an adaptive response to changed environmental conditions, are, in some instances at least, partially or completely reversible. The "fixed virus" of rabies, for example, has been observed to revert partially to the characteristics of "street virus" by serial passage in the sciatic nerve of rabbits,[43] and viscerotropism has been restored to a neurotropic strain of yellow fever virus by intrahepatic passage in the monkey.[39] This reversion of the yellow fever virus has also been found to occur apparently spontaneously,[44] a matter of some practical importance since a variant strain of this type is used for human immunization.

The interpretation of the phenomena of variation in the viruses presents almost precisely the same difficulties as the interpretation of similar phenomena in bacteria. This matter has been discussed at length elsewhere (p. 187) and need not be considered here.

Immunity to Virus Diseases.[45] Recovery from some virus diseases, *e.g.* measles and yellow fever, is accompanied by the development of solid and lasting immunity, but others, such as influenza, produce only a temporary resistance. There is no reason for doubting that immunity in virus diseases is of the same general nature as that seen in other infections, but the intracellular position of the virus introduces other factors which deserve special attention.

Active immunity may be produced artificially in a number of ways, including inoculation with virulent virus, with attenuated virus, or with virus treated with bactericidal agents. The inoculation of virulent virus, either by an unnatural route or in conjunction with antiserum injected separately or mixed with the virus, often produces a solid immunity comparing favorably with that resulting from clinical infection. Intramuscular inoculation of fully virulent psittacosis virus has been used for the immunization of laboratory workers; the normal portal of entry is the respiratory tract and disease does not result from the artificial inoculation. Hogs are usually immunized against hog cholera by

[40] Stuart-Harris: Lancet, 1939, *i*:497.
[41] Andrewes and Shope: Jour. Exp. Med., 1936, *63*:157.
[42] Berry and Dedrick: Jour. Bact., 1936, *31*:50.
[43] Nicolau and Kopciowska: Compt. Rend. Soc. Biol., 1935, *119*:140.
[44] Findlay and MacCallum: Brit. Jour. Exp. Path., 1938, *19*:384.
[45] *Cf.* the reviews of Bedson: Proc. Roy. Soc. Med., 1937, *31*:59; Burnet, Keogh and Lush: Australian Jour. Exp. Biol. Med., 1937, *15*:227; 3rd lecture in Rivers: *Viruses and Virus Diseases,* Stanford Univ. Publ. Ser. Med. Sci., 1939, Vol. 4, No. 1.

the simultaneous injection of virus-containing blood in one groin and serum in the other. The inoculation of virulent virus, is, however, an inherently dangerous procedure and one that is seldom applied to man though frequently used in the laboratory.

Another method of immunization is the inoculation of a strain of virus attenuated by adaptation to another host. Vaccinia is perhaps to be regarded as variola attenuated by animal passage, and the inoculation of man with vaccine virus provides a solid immunity to smallpox. Similarly, rabies prophylactic is rabies virus attenuated for man by adaptation to the rabbit, and recently developed methods for active immunization against yellow fever make use of a virus strain adapted to tissue culture.

The problem of producing effective immunizing preparations in which the virus is entirely non-infective is complex. At one time it was thought that such preparations would not produce the immune state, a belief probably based on the fact that it was impossible to introduce enough virus antigen into the host to induce a detectable immune state except by injecting active virus and letting it multiply there. Since methods have been discovered for producing large amounts of virus, such as by cultivation in fertile eggs, it has been possible to produce effective antigens in which the infectivity has been entirely destroyed. Care must be exercised in the selection of an inactivating agent to avoid using one that destroys the antigenicity as well as the infectivity. Formalin and ultraviolet radiation have been most satisfactory to date for preparing viral vaccines; the former is used in equine encephalomyelitis and influenza vaccines, and the latter has proven of value experimentally with several viruses.

There is some evidence that the lasting immunity following a virus disease, when it does occur, is due to an infection-immunity (p. 740). Submaxillary gland virus persists as an inapparent infection in guinea pigs, vaccinia virus has been recovered from rabbits as long as 252 days after infection, and viruses persist indefinitely in plants as an inapparent infection. Other work has failed to show a correlation between persistence of *detectable* vaccinia virus and immunity.[46] That virus may persist in at least some cases in association with the immune state would appear, however, to be definitely established. Obviously a vaccine made with an inactive virus cannot be expected to produce lasting immunity if the latter is dependent upon persistence of living virus.

Humoral antibodies are developed as a consequence of immunization with the viruses as evidenced by the protective properties of immune serum. The *serum neutralization test,* the most commonly used immunological reaction in the study of the virus diseases, consists of the inoculation of a susceptible animal with a mixture of virus and serum; immune serum will protect against many lethal doses of virus. The nature of the protective process is not clear.[47]

The antibody content of antiviral sera may be demonstrated in other ways. The agglutination of elementary bodies by immune serum may be observed in the case of the larger viruses both microscopically and macroscopically in the same manner as the agglutination of bacteria.[48] Precipitin tests in vaccinia and variola were early described, and in the case of vaccinia the flocculation

[46] *Cf.* Pearce: Jour. Inf. Dis., 1940, 66:130; Morgan and Olitsky: Jour. Immunol., 1940, 39:1.
[47] *Cf.* the discussion by Salaman: Brit. Jour. Exp. Path., 1938, 19:192.
[48] Craigie and Wishart: Brit. Jour. Exp. Path., 1934, 15:390.

has been found to be due both to a true precipitation of soluble antigen and to an agglutination of elementary bodies.[49] Precipitin tests with specific immune sera have been described with the soluble antigens of lymphocytic choriomeningitis and of infectious myxoma. The complement-fixation test has been reported to be positive in a variety of virus diseases, including vaccinia, lymphocytic choriomeningitis, equine encephalomyelitis, yellow fever, influenza, lymphogranuloma venereum and others. Of the *in vitro* serological tests, complement fixation is perhaps best adapted to the study of the virus infections since relatively small amounts of antigen are required.

The contribution of humoral antibody to immunity against virus infections is variable from one disease to another. There is reason to believe that antibody is ineffective against intracellular virus, and it would seem to follow that once a virus becomes established in the tissues considerable multiplication could occur in spite of antibody being present in the blood and tissue spaces. Indeed, in the unique situation found with virus tumors, it appears that the virus is present in each daughter cell following mitosis and, multiplying there, stimulates the daughter cell in turn to growth and division. Such a virus is capable of indefinite multiplication (as long as the tumor is growing) without ever being outside a cell.

The question of passive immunization is of interest in this connection. There is an analogy with certain of the bacterial toxemias, in that antibodies are unable to protect cells already parasitized but the presence of antibody may serve to prevent the further dissemination of infection. Practically, there is reason to think that virus may be widespread in a tissue and established in many cells before clinical signs of infection are apparent. The therapeutic effect of antiviral sera in general is not great.

Hypersensitivity. A hypersensitive state has not been demonstrated in most of the virus diseases. In venereal lymphogranuloma, however, a skin reaction is produced in response to the intradermal inoculation of inactivated virus (p. 883). A skin test has been used also for detection of allergy to the mumps virus (p. 875).

Antigenic Analysis of Viruses. With some of the smaller viruses, and those shown to consist of a single molecular species, it appears that a single antigen is responsible for the various serological reactions which may be demonstrated, such as neutralization, complement fixation and precipitation. Study of some of the more complex viruses has shown that soluble antigens demonstrable by complement fixation or precipitation technique may be separated from the virus itself. Indirect evidence of duality, at least, of antigens is obtained from a comparison of immunologic relationships among groups of viruses when tested in different ways. The pattern of relationship has been found to be quite different in some cases, depending upon which test is employed. For example, in the psittacosis group (p. 876), the complement-fixation test reveals an antigen common to the group, while in contrast, a serum neutralization technique has indicated strain specificity.

The most detailed examination of the antigenic make-up of a virus has taken place in Rivers' laboratory in a study of vaccinia virus.[33] A soluble antigen first described by Craigie in 1932 has been found to be protein in

[49] Craigie: Brit. Jour. Exp. Path., 1932, *13*:259.

nature and to consist of two components (L and S) with different heat stabilities. An "X" antigen, responsible for agglutination of elementary bodies, is a complex antigen, part of which may be nucleoprotein. In addition, there is an antigen of unknown nature responsible for the production of neutralizing antibody.

Non-Specific Factors in Resistance. Age is a factor in resistance or susceptibility to many infectious agents, but several factors associated with age appear to be unique in influencing the pathogenesis of virus infections. Sabin[50] and his colleagues found that in young mice and guinea pigs one of several neurotropic viruses, inoculated intramuscularly or by other peripheral routes, was able to progress to the central nervous system and produce infection there. As the animals became older certain barriers appeared so that virus no longer was able to reach the central nervous system after peripheral inoculation. The myoneural junction is apparently the site of one of these barriers, and others occur in the anterior rhinencephalon and the blood vessels. In this study, age apparently did not influence susceptibility to intracerebral injection, but it is known to do so in the case of rabies virus in mice and St. Louis encephalitis virus in rats.

When one considers that a virus is intimately dependent for its multiplication upon living host cells, it is not surprising to find that disturbances which affect the state of the cells or tissue may influence profoundly their susceptibility to virus infection. One of the most clear cut examples of this is found in the observation[51] that nerve cells are refractory to poliomyelitis virus for a time after section of their axons. A profound disturbance of the cell body occurs after such section, as evidenced by the reaction of chromatolysis, and, although the refractiveness does not exactly parallel this visual evidence of an altered condition, the insusceptibility must depend upon what may be called a change in physiologic state of the cell.

Susceptibility of cells is also influenced by temperature. It is well known that fever, artificial or otherwise, will often bring on an attack of herpes (p. 862). Whether this is a direct effect of heat on the susceptible cells is unknown. Relatively small differences in temperature of experimental animals or tissue cultures sometimes determine whether growth of a virus will occur.

A number of investigations concerning the influence of *vitamins* upon resistance to virus infection have been reported. Thiamine deficiency in pigeons apparently activated a latent virus of the ornithosis group.[52] Vitamin deficiencies may retard the development of the natural barriers to virus migration described above. Observations with rodent-adapted poliomyelitis virus indicated that thiamine deficiency rendered mice more resistant to that virus, and that simple undernutrition had a similar effect. This is further discussed on page 223.

Numerous investigators have attempted to find chemical or antibiotic agents that would be effective against viruses. The search has been almost entirely unsuccessful except for the finding that the agents of the psittacosis-lymphogranuloma venereum group are susceptible to a number of therapeutic agents including the sulfonamides, penicillin and chloromycetin. This and other char-

[50] Sabin and Olitsky: Proc. Soc. Exper. Biol. Med., 1938, 38:597.
[51] Howe and Bodian: Bull. Johns Hopkins Hosp., 1941, 69:92.
[52] Pinkerton and Swank: Proc. Soc. Exp. Biol. Med., 140, 45:704.

acteristics of this group indicate their close relation to the bacteria, and, indeed, some workers would exclude them from the "true viruses." In addition to the chemical substances that might be expected to have an antiseptic action, there has been an attempt, especially with influenza virus, to find some substance that would prevent adsorption of the virus to the cell by competing with the normal receptor substance of the cell for attachment to the virus (p. 867).

Attention has also been directed to the use of some substance that would interfere with virus growth in the cell by rendering the cell less suitable as a "culture medium" for the virus—that is, bringing about an unfavorable condition for the virus similar to that induced by inadequate nutrition. Only slightly encouraging results have thus far been achieved.[53]

Interference Phenomenon. The presence of one virus in a susceptible tissue sometimes prevents the growth of a related strain in the same tissue. A number of examples of this phenomenon have been recorded and the evidence is strong that it does not depend upon the mechanisms usually associated with the immune state. In 1935 Hoskins[54] reported that inoculation of a monkey with both virulent pantropic yellow fever virus and an avirulent neurotropic strain did not result in infection, provided the neurotropic strain was injected not later than twenty hours after the virulent strain was given. Similar observations have been reported for several other combinations of animal viruses and for plant viruses. Influenza virus[55] and bacteriophage[56] when treated with ultraviolet light to the point of destroying infectivity were still able to exert the interfering effect upon the same or related active strain.

The Nature of Viruses (Summary). For many years the filterable viruses were regarded as exceedingly small microorganisms differing only in size from bacteria. Proliferation and adaptive responses to altered environmental conditions are reasonably regarded as properties possessed by living as opposed to inanimate matter. There is, in fact, direct microscopical evidence that the elementary bodies of some viruses multiply by binary fission.

The obligate cytotropism of the viruses is perhaps to be regarded as evidence of a highly developed degree of parasitism approached by certain bacteria such as the leprosy bacillus, Bartonella, the spirochete of syphilis and others. The failure of the viruses to exhibit an independent metabolism has led many to believe that these agents are living microorganisms so degenerate that they are dependent upon the host cells for enzyme systems and are in the nature of "naked nuclei" or genes which adopt the protoplasm of the host as their own cytoplasm.[57]

In recent years the demonstration, by filtration experiments and other methods, of the exceedingly small size of some viruses, together with the isolation and study of the "virus proteins" of some of the plant viruses, has led many to question the animate nature of these agents. In the case of a very small

[53] Thompson: Jour. Immunol. 1947, 55:345; Cutting *et al.*: Jour. Immunol., 1947, 57:379.
[54] Hoskins: Am. Jour. Trop. Med., 1935, 15:675.
[55] Ziegler and Horsfall: Jour. Exp. Med., 1944, 79:361.
[56] Luria: Arch. Biochem., 1942, 1:207.
[57] Cf. the discussion of Gortner: Science, 1938, 87:529.

virus, 10 to 15 mμ in diameter, there cannot be any great degree of organization, for there is room for only a few protein molecules. The "virus proteins" are regarded by many as autocatalytic substances lying in the borderland between the lifeless and the living.

Clearly, then, no final conclusion can as yet be drawn regarding the nature of the viruses. Two points should be noted in this connection. In the first place, the distinction between animate and inanimate matter may perhaps become quite as pointless as regards the viruses as the once debated plant or animal nature of the bacteria. In the second, it is not only unnecessary but undesirable to assume that all viruses are of essentially the same nature; it is altogether possible, even probable, that some of the larger ones, such as the virus of vaccinia, are minute microorganisms which differ from bacteria only in size and cultural requirements, while very small viruses are homogeneous autocatalytic proteins.

Chapter 37

VIRUS DISEASES OF MAN[1]

By F. B. Gordon, Ph.D., M.D.

Virus infections of man produce many different clinical types of disease, and their heterogeneity in regard to this character as well as others exceeds that of the true bacterial diseases. Many of the most important infectious diseases of man are in this group, and although the technical difficulties of virus investigation have interfered with the accumulation of knowledge concerning some of these diseases, intensive investigation in other cases has uncovered sufficient facts to lead to valuable methods of control. The practical difficulties of propagation and identification of a virus, in contrast to a bacterium, are of particular significance in the laboratory diagnosis of the infections. In many cases there is no satisfactory method of laboratory diagnosis that can be used routinely, although several notable exceptions occur. With continued advance in knowledge the development of satisfactory methods for laboratory diagnosis of more and more virus diseases may be expected.

In this chapter pertinent data concerning the virus diseases of man are brought together. Many of these viruses also produce spontaneous disease in animals. For information concerning virus diseases affecting only animals or plants the reader is referred to appropriate general references in Chapter 36.

SMALLPOX (VARIOLA) AND VACCINIA

Smallpox is a generalized disease characterized by skin lesions appearing first as papules and developing into vesicles and pustules which, after healing, may leave the deep, pitted scars that sometimes accompany recovery from this disease. Smallpox was recognized in China before the Christian era, and during the Middle Ages it was prevalent throughout Europe, reaching England and spreading to the New World in the sixteenth century. At the present time it is endemic in many parts of the world and epidemics are not infrequent, especially when prophylactic measures are relaxed.

Epidemiology.[2] Smallpox is usually most prevalent in the winter or early spring months. It attacks all age groups but has appeared at times mainly as a disease of children, or at other times as a disease of adults, depending upon the level of immunity, naturally or artificially acquired, in the population at the time.

---

[1] Detailed information on many of these diseases may be found in Rivers: *Viral and Rickettsial Infections of Man.* J. B. Lippincott Company, Philadelphia. 1948; and in van Rooyen and Rhodes: *Virus Diseases of Man.* Thos. Nelson & Sons, New York. 1948.

[2] *Cf.* Russell: *Epidemiology and Control of Variola,* pp. 176–200 (See Rivers, ref. 1).

The infectivity of a case is high and lasts from some time during the incubation period until well into convalescence. Virus is present in the skin lesions, remaining active for long periods in the dried crusts, and is no doubt also present in respiratory and buccal discharge, especially when lesions are present upon the mucous membrane of these regions. Transmission is by direct or indirect contact, and aerial convection is strongly suspected.[3]

Observers have suspected for many years that smallpox exists in two forms—a severe "malignant smallpox," and a benign type which has been called by a variety of names (alastrim, cottonpox, Kaffir pox, paravariola, etc.). Convincing epidemiological evidence of the existence of two forms of the disease has
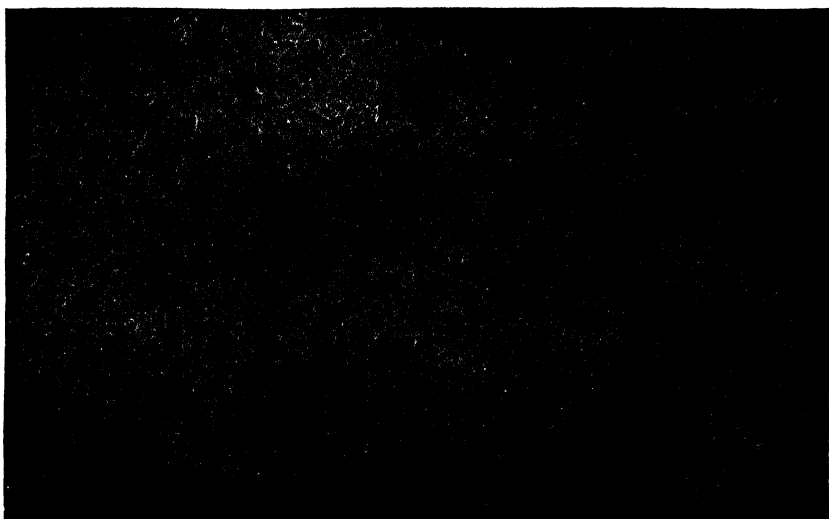
Fig. 242. Elementary bodies of vaccinia; Morosow's stain; × 1000. From Parker and Rivers: Jour. Exp. Med., 1935, 62:65. (Reproduced through the courtesy of the authors and publisher.)

been presented by Chapin[4] and by Hedrick.[5] The case fatality in the mild form is less than 1 per cent; that in the malignant form 25 to 30 per cent. There is no evidence that there is a mutation of one strain of the virus to another. The mild form is the predominant type in this country at present, although the malignant form has occurred from time to time, apparently as the result of importation. Due to its mildness the former type is actually the more difficult to control, and there have been wide fluctuations in the number of cases reported in the Registration Area. In 1938 there were 14,939 cases (11.5 per 100,000) but in 1947 the figure was only 173. The death rate has remained less than 0.1 per 100,000.

Vaccinia. Some of the early investigators found that the agent of smallpox, when inoculated into calves or rabbits, was changed in nature and became the agent of vaccinia, used in immunization against smallpox. A preliminary

[3] Millard: Brit. Med. Jour., 1944, 1:628.
[4] Chapin: Jour. Prev. Med., 1932, 6:273.
[5] Hedrick: Pub. Health Repts., 1936, 51:363.

passage through monkeys is said to facilitate this modification.[6] Vaccinia in man may be defined as a mild, usually local, disease transmitted by artificial dermal inoculation, and resulting in immunity against smallpox. Investigations of these two infections have gone hand in hand, and it is impracticable, if not undesirable, to treat them separately.

The Virus. Although bacteria may often be found in variolous pustules, their irregularity of presence and type led the early observers to look further for an etiological agent. Attention was drawn to peculiar bodies within the epithelial cells of lesions of both variola and vaccinia, which Guarnieri in 1892 regarded as protozoa and named *Cytoryctes vacciniae et variolae*. These are now recognized as inclusion bodies (Guarnieri bodies). Paschen later described tiny corpuscular elements (elementary bodies) in vaccine lymph, which are now known to be particles of virus (p. 842 and Fig. 242). Numerous morphological studies have been made of Guarnieri bodies and vaccinal elementary bodies. Various observers have brought evidence that the Guarnieri body is made up of a colony of elementary bodies which are released when the cell and the contained Guarnieri body eventually are disrupted. This is the process depicted with another virus in Fig. 235. The chemical composition of this virus has been described on page 849 and its complex antigenic structure on page 854. The appearance of the elementary bodies under the electron microscope is shown in Figs. 238 and 239.

The virus of smallpox is said to be naturally infectious for monkeys as well as man, and if it is transmitted serially through monkeys it apparently keeps its identity, in contrast to the modification produced by passage in calves or rabbits. It also retains the character of variola virus when passed in fertile eggs, in which it has been propagated by several workers.[7] Vaccinia is transmissible to many species of animals, but varying degrees of reaction are obtained. The rabbit is the common laboratory animal for the maintenance of this virus, and strains may be propagated in the skin, testis or brain. The inoculation of variolous material onto the cornea of a rabbit (Paul's test) has been used as a diagnostic procedure for smallpox. The development of a keratitis in which Guarnieri bodies are demonstrable microscopically indicates a positive test (Fig. 232). The demonstration of Paschen bodies in skin scrapings by stained smear has also been suggested for diagnosis.[8]

Immunization against Smallpox. Immunization against this disease, practiced from early times, was first accomplished by variolation, an artificial inoculation with smallpox virus. Crusts of smallpox lesions or the fluid from vesicles was applied in one of several ways to the skin or the nasal mucous membrane. The result was usually a mild case of smallpox with recovery, and the subject no doubt enjoyed a high degree of immunity against the natural disease.

The relation between human smallpox and cowpox, a disease manifested under natural conditions as a vesicular eruption on the udder, was clearly established in 1798 by the well-known observations of Jenner. Noting that dairy workers who had had cowpox were less liable than others to contract smallpox,

---

[6] Attempts to produce the transformation are by no means always successful. For a review and account of a successful attempt see: Horgan: Jour. Hyg., 1938, 38:702.

[7] *Cf.* Nelson: Jour. Exp. Med., 1939, 70:107.

[8] van Rooyen and Illingworth: Brit. Med. Jour., 1944, 1:526.

Jenner advocated on this and other substantial grounds the systematic inoculation of cowpox virus as a protection against smallpox. The practical success achieved by this method of immunization was amply confirmed and is now a matter of common knowledge.

The Relation of Cowpox to Vaccinia. The terms cowpox and vaccinia are usually used synonymously since the lesions produced by the two are quite similar grossly, and it has been shown repeatedly that strains of the two infections produce very good cross immunity. The ultimate origin of all strains of vaccine virus now used commercially is not known. It is assumed that some at least have been derived from variola, while others may represent the original cowpox strain used by Jenner.

Observations by Downie[9] have added evidence to previous suggestions that these two viruses are not identical. He has described differences in the histological appearance of the lesions and inclusion bodies of the two viruses when grown upon the chorioallantoic membrane of chick embryos and in animals. What is perhaps more significant, cross-serological tests (agglutination, complement fixation, neutralization) with absorbed and unabsorbed sera showed that there exists a qualitative antigenic difference between the two types. Besides cowpox, there are a number of other "pock diseases" of animals which, it is thought, are related more or less closely to cowpox and variola. Immunological tests by the refined techniques now available would further an understanding of relationships within this group.

Vaccination. Vaccine for human use is prepared by rubbing the virus into scratches on the shaved and carefully prepared abdomens of calves. The "seed virus" used for inoculation is kept fully active and at high titer by frequent passage through rabbits. After confluent vesicles have appeared along the scratches the virus is harvested by scraping off the vesicle walls and contents (vaccine "lymph") and emulsifying in glycerin. Bacterial counts are made, and cultural and animal tests are performed to detect *Cl. tetani* or other pathogens. After satisfactory tests for potency of the virus, the vaccine is tubed and stored at refrigerator temperatures.

Vaccination by the scratch method was popular at one time, but the method in favor at present is known as the "multiple-pressure" method,[10] which has the advantage of producing minimal scarring and being apparently less liable to bacterial infection. After the site is cleansed with acetone, a needle held horizontal to the surface is pressed repeatedly against the skin through a drop of vaccine, the elasticity of the skin causing the point of the needle each time to enter the epidermis. The excess vaccine is then wiped off and a dressing is not used.

A successful vaccination in a non-immune person results in the production after two or three days of a papule which develops into a vesicle, reaching its maximum on the eighth to twelfth day, and followed by a crust which finally drops off, leaving the characteristic scar. The "immune reaction,"[10] seen in immune persons, consists in the rapid development of a papule which begins to subside on the second or third day and never reaches vesicle stage. An "accelerated" or "vaccinoid reaction," probably indicating partial immunity, is

[9] Downie: Jour. Path. Bact., 1939, 48:361.
[10] Leake: Pub. Health Repts., 1927, 42:221.

similar to the true or "primary" reaction described above except that it is not so severe and runs a shorter and more rapid course.

In addition to calf lymph, tissue-culture virus,[11] virus grown in fertile eggs,[12] and filtered sheep-vaccine pulp[13] have been suggested and used to some extent for jennerian prophylaxis. They have the advantage of being bacteria-free, which allows intradermal inoculation followed by immunity without the production of a scar.

Vaccination is recommended for infants during the first year of life. A first vaccination delayed until after the first year apparently increases the chances of "postvaccination encephalitis" (p. 903). A second vaccination during school age or later is regarded as providing a relatively safe level of immunity for life.

HERPES

Infection in man with the virus of herpes[14] is manifested by the appearance of a vesicular eruption on skin or mucous membrane. Often located at the

Fig. 243. Intranuclear herpetic inclusion in human brain. Phloxine and methylene blue stain; × 1075. (Zarafonetis, *et al.*: Amer. Jour. Path., 1944. Courtesy Army Institute of Pathology, Accession No. 85691.)

borders of the lips or nares ("cold sores," *herpes labialis*), the vesicles are sometimes distributed bilaterally or may be more widespread. The mucosa of the genital tract may be attacked (*herpes genitalis*), and keratitis has been associated with this virus. Herpes often accompanies fevers of various types, particularly pneumonia and cerebrospinal meningitis ("fever blisters," *herpes febrilis*). In some individuals exposure to cold, mild respiratory tract infection, emotional strain or menstruation will incite a crop of herpetic eruptions, while

[11] Rivers, Ward and Baird: Jour. Exp. Med., 1939, 69:857.
[12] Buddingh: Amer. Jour. Pub. Health, 1937, 27:1135.
[13] Henderson and McClean: Jour. Hyg., 1939, 39:680.
[14] The disease described here is also called *herpes simplex* to distinguish it from *herpes zoster*, another disease which may also be of virus etiology.

in others the stimulus must be more severe. Boak, Carpenter and Warren,[15] in work with artificial-fever therapy for syphilis and other infections, reported that about 60 per cent of the subjects suffered an attack of herpes simplex. Other observations[16] have shown that aphthous stomatitis, a common clinical condition in infants from one to three years of age, often accompanied by fever and toxic symptoms, is caused by the virus of herpes, and such episodes are regarded as the result of the first exposure of the child. Later manifestations are much milder, *e.g.*, fever blisters. Herpes virus has been repeatedly demonstrated in human saliva.

The questionable relation between herpes and encephalitis lethargica is discussed elsewhere (p. 895), but in any case, this virus has been isolated from a number of isolated cases of fatal human encephalitis.[17] Intranuclear inclusions of the herpetic type were demonstrated in brain tissue of the patients and immunologic studies proved the identity of the virus. It is now clear that this virus can on occasion give rise to encephalitis in man.

The Virus. Transmission was demonstrated in 1912 when Grüter showed that fluid from herpetic vesicles produced a keratitis when placed upon the scarified cornea of the rabbit. Herpetic lesions may also be produced in the rabbit's skin, and from both these sites virus may migrate along nerve pathways to the brain, producing an encephalitis.[18] Other animals exhibit varying degrees of susceptibility. A local vesicular lesion is produced after inoculation of the foot pad of a guinea pig, but the central nervous system is usually not invaded. An encephalitis follows intracerebral inoculation of mice, and generalized infection of suckling mice has been described[19] following intranasal inoculation. The results of attempts to infect monkeys have been variable. Multiplication of the virus is easily obtained in tissue culture as well as upon the chorioallantoic membrane of the chick embryo.[20]

The characteristic intranuclear inclusion (type A), seen in infected tissues, is an acidophilic mass which almost completely replaces the nuclear chromatin. The latter accumulates at the nuclear membrane, a disturbance frequently called margination of the chromatin (Fig. 243; see also Fig. 234 for this type of inclusion). Some workers have reported the demonstration of elementary bodies in infected tissues, or within the inclusion body.[21]

Herpes virus is relatively large, falling within the group having a diameter greater than 100 mμ. It may be stored for many months in infected tissue in 50 per cent glycerin, and some investigators have reported an enhancement of virulence by such treatment.

Immunity. Although some local immunity may follow the skin lesions in man, autoinoculation in another region produces infection, and apparently little or no general immunity occurs. Some persons are especially prone to herpes, and it has been postulated that the virus is latent at some focus within their bodies, manifesting its presence at intervals by the appearance of the

[15] Boak, Carpenter and Warren: Jour. Bact., 1934, 27:83.
[16] Dodd, Johnston and Buddingh: Jour. Pediat., 1938, 12:95.
[17] Whitman, Wall, and Warren: Jour. Am. Med. Assn., 1946, 131:1408.
[18] Goodpasture and Teague: Jour. Med. Res., 1923, 44:139.
[19] Slavin and Berry: Jour. Exp. Med., 1943, 78:321.
[20] Shaffer and Enders: Jour. Immunol., 1939, 37:383.
[21] Nicolau and Kopciowska: Ann. Inst. Pasteur, 1938, 60:401.

eruption after fever or other suitable stimuli. Although the skin areas involved are usually regarded as the site of the latent virus, its demonstration in such sites has not been successful. Recognizing the transmission of the virus along nerve tracts, the Gasserian ganglion was considered a likely site of latency but tests by subinoculation of animals were unsuccessful.[22] Herpes virus has been demonstrated in saliva and spinal fluid of persons with and without herpes lesions, but the significance of these findings, in regard to carriage of the virus, is uncertain.

The results of neutralization tests with human serum show that most persons (60–90 per cent) posses antibodies against the virus of herpes. Several observers have noted a peculiar "all-or-none" result with human sera—either they have a high titer, or they are devoid of antibody. There is some correlation between the presence of antibody and a tendency to recurrent herpes, suggesting that the antibody in man is the result of persisting latent infection.

Herpes in animals is often followed by a general immunity, although sometimes of low grade. Immunity of the cornea of a rabbit following herpetic keratitis is said to last several months but finally to disappear. Immunity in the brain persists longer. Complement-fixing as well as neutralizing antibodies are present in the sera of hyperimmunized animals. Immune serum has been reported to protect mice and rabbits against the development of encephalitis after peripheral inoculation of virus, but this was not possible when virus was introduced into the brain.[23]

The herpes virus is considered to have been highly successful in its adaptation to man. It is found in high incidence in most parts of the world but, almost without exception, produces the mildest kind of illness, and "lives with" its individual host indefinitely.

Two other similar viruses are found in animals, and are regarded as counterparts of herpes virus in man. *Pseudorabies*[24] is a mild disease of swine, highly contagious by means of nasal secretions. Like herpes, when transmitted to the rabbit or other experimental animal, a severe fatal infection of the nervous system occurs. A similar phenomenon occurs naturally in cattle when in contact with infected swine, and is called *mad itch*.

A less studied virus is the so-called *B virus*[25] which was isolated from the central nervous system of a laboratory worker who died of an ascending myelitis with visceral necrosis following a bite on the hand by an apparently normal monkey. The virus is infectious for rabbits and monkeys and is capable of producing lesions in skin, viscera and nervous tissue, as is the pseudorabies virus. B virus has shown partial antigenic relationship to both herpes and pseudorabies virus.[26] A number of normal monkey sera have been found capable of neutralizing B virus and it is postulated, therefore, that natural infections occur in monkeys.

The natural history of these three viruses is discussed by Burnet.[27]

[22] Burnet and Lush: Lancet, 1939, *i*:629.
[23] Hallauer: Ztschr. f. Hyg., 1937, *119*:505.
[24] Shope: Jour. Exp. Med., 1935, *62*:85.
[25] Sabin and Wright: Jour. Exp. Med., 1934, *59*:115.
[26] Sabin: Brit. Jour. Exp. Path., 1934, *15*:248.
[27] Burnet, *Virus as Organism.* Harvard University Press, Cambridge, Mass. 1945.

INFLUENZA[28]

Influenza occurred at least as early as the fourteenth century, and since the beginning of the sixteenth century waves of this disease have swept over the world at more or less frequent intervals, the latest being the great pandemic of 1918–19, in which it is estimated that more than 21 million persons died.[29]

Etiology. The specific microorganism that was most commonly regarded as the cause of influenza following the pandemic in 1890 is the hemophilic bacillus discovered by Pfeiffer (*Hemophilus influenzae*, p. 515). This bacillus, although not found until 1892, after the initial wave of true influenza had passed, was generally regarded by bacteriologists up to 1918 as the causal agent
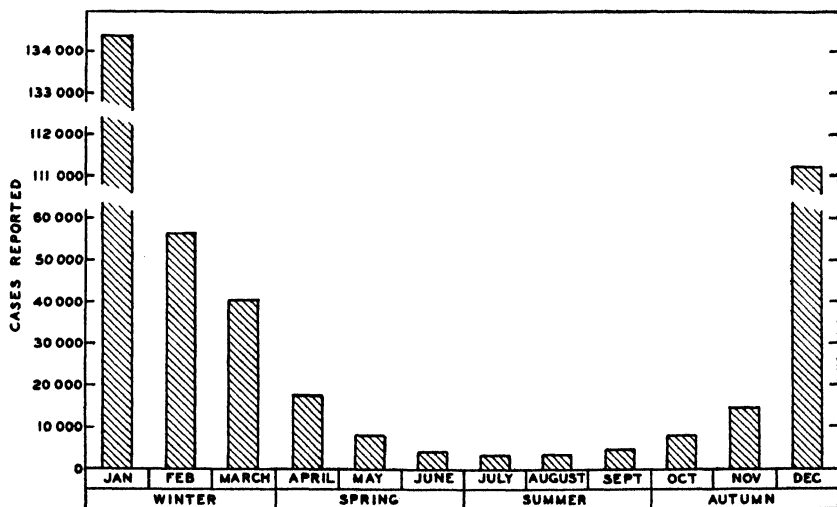
Fig. 244. The seasonal incidence of influenza. Averages of reported cases by months for the years 1939 to 1945 inclusive. Data from Supplements to Public Health Reports.

of influenza. As a consequence of the bacterial observations made in 1918–20 and later, it is now established that *H. influenzae,* although often present as a secondary invader in influenza, is not the primary agent. A great variety of other microorganisms, such as streptococci, especially of the green-producing type, pneumococci, staphylococci, *Neisseria catarrhalis,* Friedländer's bacillus, and various nondescript microorganisms, are found similarly associated with cases of influenza both in the upper respiratory tract during the attack and in the pathological lesions after death.

The Virus. Many of the features of influenza had suggested that this disease was caused by a filterable virus, but up until 1933 experimental transmission of the infection with filtered material had been successful only with

[28] *Cf.* reference 27; also Horsfall: *Virus Diseases.* Cornell University Press, Ithaca. 1943.

[29] For a review of the earlier literature, see Jordan: *Epidemic Influenza.* Amer. Med. Assn., Chicago. 1927.

chimpanzees.[30] The work of Smith, Andrewes and Laidlaw[31] (1933), who succeeded in establishing the disease in ferrets by intranasal inoculation with nasopharyngeal washings, marks the beginning of intensive and productive investigation of this disease. Influenza in ferrets manifests itself by fever, inflammation of the nasal mucosa, and sometimes pneumonia, and transmission is accomplished by intranasal inoculation with filtered respiratory discharge or tissue emulsions. It was found that ferret virus can be adapted to mice,[32] in which pneumonia appears after intranasal inoculation, and other animals have shown varying degrees of susceptibility.
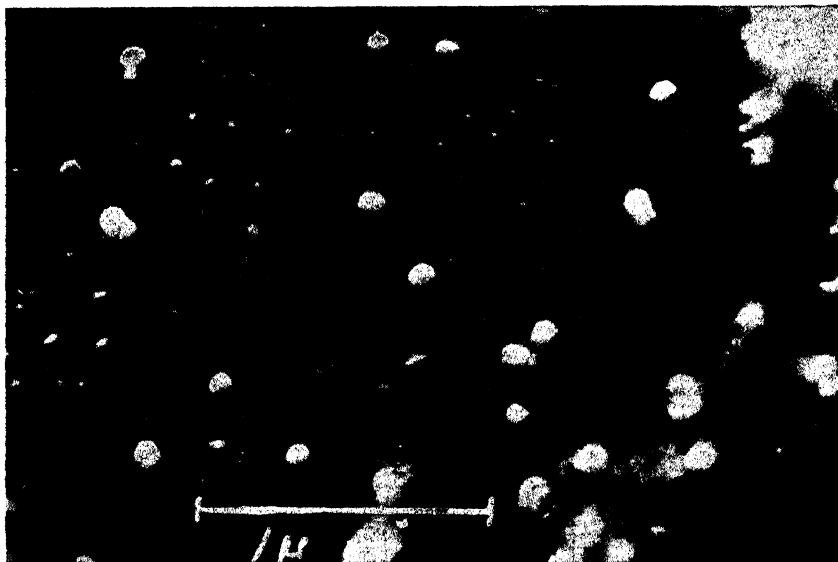
Fig. 245. Electron micrograph of a shadow-cast preparation of influenza virus. (SAB No. 136.)

Virus can be cultivated in the allantoic cavity of embryonated eggs from which it is readily harvested in high concentration by drawing off the allantoic fluid.[33] Primary isolation from throat washings is accomplished by inoculation of the amniotic or allantoic cavity. Transmission of the laboratory strains back to man has been accomplished experimentally, thus completing the evidence for the etiologic role of this virus. The strains isolated have been derived from "epidemic influenza"; the causative agent of the epidemiologically distinguishable "pandemic influenza," which has not occurred since 1918, remains unknown.

The virus may be inactivated by soaps, formalin and ultraviolet light without destroying the antigenicity. Treatment of throat washings with the proper concentration of a detergent, or with penicillin and similar drugs, does not inactivate the virus, and has been used for preventing bacterial growth during

[30] Long, Bliss and Carpenter: Jour. Amer. Med. Assn., 1931, 97:1112.
[31] Smith, Andrewes and Laidlaw: Lancet, 1933, ii:66.
[32] Francis and Magill: Science, 1935, 82:353.
[33] Burnet: Austral. Jour. Exp. Biol. Med. Sci., 1941, 19:291.

direct isolation in the egg.[34] Propylene glycol vapor will destroy influenza virus suspended in the air.[35]

A toxic effect of the virus, demonstrated by death of mice and by lymphocytopenia in the rabbit, has been reported.[36]

Early estimations of the size of the virus gave it a diameter of 80 to 120 mμ. More recent estimations based on sedimentation velocity and electron microscopy indicate sizes of 100 to 125 mμ, differing with the strains of virus used.[37] Virus emulsions are used as antigens in complement-fixation tests with immune serums. The infective particle apparently acts as antigen, but a "soluble" antigen, perhaps of protein nature, has also been demonstrated, in allantoic fluid and in infected mouse lungs.[38]

In 1941 Hirst[39] reported that influenza virus, as contained in the allantoic fluid of infected eggs, has the ability to agglutinate chicken erythrocytes mixed with it. Although certain other viruses act similarly, this observation (Hirst's phenomenon) has had its greatest application in investigations on influenza. The hemagglutinating power is closely related to infectivity, and the ability of antiserum to neutralize virus is reflected in its inhibition of agglutination of erythrocytes in the presence of virus. Thus, Hirst's observation has led to the development of an *in vitro* serological test that has been used extensively. Red cells of other species are agglutinated in varying degree.

Elution of virus from erythrocytes takes place spontaneously at an interval after adsorption, after which no more virus can be adsorbed to the cells. This and other observations have suggested that the virus has an enzyme-like action upon some specific receptor substance of the cell. An apparently analogous series of events has been observed when the epithelium of the bronchial tree is exposed to the virus, indicating a possible significance of this activity of the virus in the pathogenesis of the disease. Efforts to determine the nature of the substrate may point the way to a therapeutically effective substance which would act by competing with the cellular receptor for union with the virus particle.

Virus Strains. The strains that had been isolated up to 1940 possessed some antigenic diversity, demonstrable by cross immunity and cross neutralization tests in mice, but there was a broad general similarity. Smith and Andrewes,[40] in a study of 28 of these strains, found that many antigenic components were present but described four major antigens that served as a basis for a rough classification of the strains. Indirect evidence, however, had indicated that some outbreaks of clinically typical influenza were caused by virus strains unrelated to the known ones, *e.g.*, serum of convalescents failed to neutralize the known strains. Light was shed on this problem in 1940 when strains were isolated[41] that appeared similar in all respects to the strains previously studied

[34] Burnet and Stone: Austral. Jour. Exp. Biol. Med. Sci., 1945, 23:161.
[35] Henle and Zellat: Proc. Soc. Exp. Biol. Med., 1941, 48:544; Robertson, *et al.*: Science, 1941, 94:612.
[36] Henle and Henle: Jour. Exp. Med., 1946, 84:623, 639.
[37] Sharp *et al.*: Jour. Biol. Chem., 1944, 156;585.
[38] Wiener, Henle and Henle: Jour. Exp. Med., 1946, 83:259.
[39] Hirst: Science, 1941, 94:22.
[40] Smith and Andrewes: Brit. Jour. Exp. Path., 1938, 19:284.
[41] Francis: Science, 1940, 92:405; Magill: Proc. Soc. Exp. Biol. Med., 1940, 45:162.

except that they were completely different antigenically. Strains falling in this group are now called influenza B, (*e.g.,* Lee strain), and the earlier strains (*e.g.,* WS, PR8, Melbourne), as well as more recently isolated, antigenically similar ones, are known as influenza A.

Variation. Burnet[27] has called attention to the great mutability of influenza virus, the principal evidence being a form of variation known as O → D. When human influenza throat washings are inoculated into the amniotic cavity of an egg the virus that appears in the first passage has the property of agglutinating guinea pig red cells well, but gives only a low titer with fowl cells. Such virus is said to be in the original (O) phase, and can be retained in this phase by passage at high dilution in the amniotic sac. If lower dilutions of inoculum are used the derivative (D) phase is produced, characterized by an ability to agglutinate guinea pig and fowl cells equally well. The virus as it infects man appears to be always in the O phase, while all laboratory strains carried in mice or by allantoic passage are in the D phase. The question is obvious: whether results of studies on D phase virus can be applied directly to the disease as it occurs in man.

Immunity. An attack of influenza produces only temporary immunity and repeated attacks in the same person have occurred. Most adults have antibody in their sera against the common strains of influenza, demonstrable either by the neutralization test, complement fixation, or inhibition of hemagglutination, indicating previous contact with influenza virus. The level of antibody varies greatly from one individual to another, as might be expected, and an attack of influenza produces a transient rise in titer, which eventually returns to the previous level. This rise has been observed not only in frank cases but in individuals who have remained well during an epidemic. Although there is some correlation between amount of serum antibody and resistance to influenza,[42] it is apparent that other factors enter. In addition to antigenic differences between strains, attention has been focused on possible local factors in the respiratory tract. Abnormality of the nasal mucosa in the ferret, observed histologically, has been shown to influence susceptibility in that animal. The significance of a virus neutralizing substance found in human nasal secretions,[43] and at least related to serum antibody, is not yet known.

Active Immunization. Parenteral inoculation of experimental animals with influenza virus will protect them against a subsequent intranasal inoculation. This can be accomplished with virus rendered non-infective in a number of ways as well as with active virus. Similar inoculation of man with various virus preparations induces an increased serum antibody titer, and susceptibility to subsequent induced infection can be lowered by such procedures. Extensive field trials of influenza vaccines containing both A and B antigen, conducted in 1943 and 1945, indicated that the incidence of natural infection with either of these types had been definitely controlled by appropriate immunization programs. These vaccines were made by formaldehyde inactivation of concentrated virus preparations derived from infected allantoic fluids.[44]

This type of vaccine proved ineffective in a number of outbreaks in 1947,

[42] Rickard, *et al.:* Pub. Health Rep., 1941, 56:1819.
[43] Burnet, Lush and Jackson: Brit. Jour. Exper. Pathol., 1939, 20:377.
[44] Salk and Francis: Ann. Int. Med., 1946, 25:443.

apparently because a strain of virus A was prevalent (*e.g.*, FM-1) that had relatively little antigenic similarity to the strains used in the vaccines. When serums of persons who had received the vaccines were examined, little or no rise in antibody titer for the newly isolated strains was evident.[45]

Passive Immunization. Injection of immune serum has a protective effect against intranasal inoculations of the virus in mice and the effect is much greater if the serum is given intranasally. Favorable results have been reported[46] in an attempt to protect man during an epidemic of influenza by allowing the subjects to inhale finely dispersed droplets of immune horse serum. Inhalation of immune human serum was found in other experiments[47] not to protect human volunteers against artificial infection induced by inhalation of finely dispersed virus suspension.

Swine Influenza.[48] A respiratory disease of swine in the Midwest was observed at the time of the 1918 pandemic of influenza in man, when its similarity to the human disease was noted. The elucidation of its etiology, antedating that of human influenza, appeared in 1930, when Shope found that two agents are associated with the disease, both of which must be present to produce the typical clinical syndrome. He isolated a filterable virus and a hemophilic organism very similar to Pfeiffer's bacillus, which he called *Hemophilus influenzae suis.* The virus alone when inoculated into a pig produced a minimal, sometimes inapparent, infection termed "filtrate disease." The simultaneous inoculation of the hemophilic organism or the inoculation of the virus into a pig which was a carrier of this bacillus resulted in production of typical swine influenza. These findings suggested that the disease in man was of a similar dual nature, but subsequent investigation with human strains has shown that the virus itself is capable of producing the complete disease picture.

Swine influenza appears on midwestern farms in the autumn and spreads with great rapidity. Shope, while not denying the contagious nature of the disease, questions whether the rapid spread can be accounted for on the basis of contact, and suggests that the sudden appearance of the disease in a previously healthy herd may be due to some "exciting" cause acting in a herd in which carriers of the virus are already present. He has discovered a complex cycle[49] of transmission, involving two reservoir hosts, which represents a possible means of dissemination of the virus in a masked form throughout a herd. He has shown that lungworms (nematodes) from infected swine contain the virus. The lungworm ova are excreted in the pig's feces where they are ingested by earthworms in which the larvae appear. The cycle is completed when the earthworm is eaten by a pig, in which the adult worms develop. Virus is carried by larvae developing from ova laid by worms in an infected pig, and reaches another pig when the infected larvae within the earthworm are ingested. The development of the clinical disease, however, does not occur until some exciting cause comes into action. Artificially, an injection of *H.*

[45] Loosli, Schoenberger and Barnett: Jour. Lab. Clin. Med., 1948, 33:789.
[46] Smorodintseff, Gulamoff and Tschalkina: Zeitschr. f. Klin. Med., 1940, *138*:756.
[47] Report of Commission on Influenza, U. S. Army, April, 1944.
[48] *Cf.* Shope: *Virus Diseases.* Cornell University Press, Ithaca, 1943, pp. 85–109.
[49] Shope: Jour. Exp. Med., 1941, 74:49.

influenzae suis can act as the exciting cause but climatic conditions also may be concerned. Natural epizootics of swine influenza occur only during the winter months (October to April) and it is only during this period that experimental transmission by lungworms is demonstrable.

The virus of swine influenza is closely related to the human strains, both ferrets and mice being susceptible to swine as well as human strains, and both types of strains producing similar infections in swine. Cross-immunization tests indicate that there is some antigenic overlapping between human influenza A and swine strains.

PRIMARY ATYPICAL PNEUMONIA (VIRUS PNEUMONIA)[50]

During the years 1940 to 1942 an influenza-like disease of the respiratory tract gained general recognition, although the first observations had been recorded some years earlier. This clinical entity was first described under a variety of names but the officially suggested term "primary atypical pneumonia, etiology unknown"[51] soon came into general use.

Epidemic and sporadic cases are recognized, the former having been observed especially among army and navy personnel, among which the disease was a potentially serious cause of incapacitation. The sporadic cases in general have been more severe and presented a wide variety of clinical characters, while the epidemic form is usually milder, many cases exhibiting only an upper respiratory infection. However, mild cases are much more readily recognized during an epidemic and we have no real evidence that this epidemiological difference is correlated with etiological differences. One of the characteristics of this disease, recorded by many observers, is the relatively severe pulmonary involvement, revealed by the x-ray, which may be present when the physical signs and general condition of the patient give no hint that the lower respiratory tract is affected.

No obvious bacterial etiologic agent has been discovered in this disease and, in contrast to the usual pneumonias, these cases are not modified by the sulfonamides or penicillin. A number of non-bacterial agents have been shown to be related etiologically to sporadic cases falling within the clinical definition of primary atypical pneumonia. Thus, several of the viruses of the psittacosis-lymphogranuloma group (p. 875) produce this type of illness. Lymphocytic choriomeningitis virus (p. 885) may produce an influenza-like disease, and infection with *Rickettsia diaporica* (*Coxiella burneti*) (p. 831) may take a pulmonary form. Primary coccidioidomycosis (p. 720) also falls within the clinical description of this disease. In the epidemic cases, however, failure to isolate the agents enumerated, as well as negative serological findings, indicates that most of these cases, at least, have a different causative agent.

Numerous viral agents including those mentioned above have been isolated by various investigators during a search for the cause of primary atypical pneumonia. Varying amounts of evidence have been submitted for the etiologic significance of each but further observations are necessary before a final evaluation can be made. References to these investigations may be found in the report by Eaton and his colleagues who have attacked this problem with some

[50] *Cf.* review by Reimann: Medicine, 1947, 26:167.
[51] War Medicine, 1942, 2:330.

success. They describe the isolation of a virus (AP) in cotton rats by inoculation of the sputum from patients. This agent is also infective for hamsters and can be propagated in the egg by inoculation into the amniotic cavity.[52]

The development of neutralizing antibody against this virus in patients with atypical pneumonia provides weighty evidence for its etiologic role. Comparing acute phase and convalescent sera, significant antibody rise was observed in 62 per cent of 84 persons with atypical pneumonia, and in 19 per cent of 77 with undifferentiated upper respiratory infection.[53]

The production of respiratory disease in human volunteers by inhalation of filtered nasopharyngeal washings of patients is not entirely convincing because of the appearance of similar disease in some of the controls that had been given autoclaved material.

Another group of investigators[54] offer evidence that an "indifferent" streptococcus (MG) plays some role in this disease. Although antibody against this streptococcus may be found in the serum of patients the evidence for its etiologic role is incomplete.

"Cold" Hemagglutinin. An interesting observation was made by Peterson, Ham and Finland,[55] and confirmed by others, that serum from cases of primary atypical pneumonia will agglutinate homologous or group O human erythrocytes in the cold. The reaction is demonstrable at 4° C. but not at 37° C., the agglutination being reversible as the tubes are warmed. The autohemagglutinin titer of the patient's serum begins to rise during the second week of illness and persists for a month or more. This reaction, although described for a number of other conditions also, is of value in diagnosis. However, the incidence of positive tests varies greatly in the different series studied, but has been reported as high as 90 per cent in some studies. It is apparently negative in those cases due to viruses of the psittacosis group.

THE COMMON COLD

One of the chief causes of disability the world over is the acute affection of the upper respiratory tract known as the common cold. The symptoms are diverse and need little description since they are experienced by most persons two or three times a year. The "sickness surveys" usually list more persons temporarily incapacitated from this cause than from any other.

Etiology. Few beliefs are more firmly fixed in the popular mind than that atmospheric changes, especially sudden drops in temperature, are responsible for outbreaks of colds. Two investigators[56] who spent eleven months studying the epidemiology and bacteriology of colds in Spitzbergen, an isolated Arctic community, cut off completely from the outside world for several months each year, noted that the arrival of the first boat of the shipping season was followed by a sudden epidemic of colds which involved the whole community in a short period of time. Other types of study have given evidence for the influence of weather.[57] It is evident that changes in weather conditions,

---

[52] Eaton, Meiklejohn and Van Herick: Jour. Exp. Med., 1944, 79:649.
[53] Eaton and Van Herick: Amer. Jour. Hyg., 1947, 45:82.
[54] Thomas, et al.: Jour. Clin. Investig., 1945, 24:227.
[55] Peterson, Ham and Finland: Science, 1943, 97:167.
[56] Paul and Freese: Amer. Jour. Hyg., 1933, 17:517.
[57] Brown, et al.: Jour. Immunol. Virus Res. & Exp. Chemother, 1945, 50:161.

872 Virus Diseases of Man

whatever effect they may have in promoting an increased susceptibility, are not the necessary primary exciting cause of an epidemic of colds. Certain puzzling epidemiological factors in the dissemination of colds without apparent contact may be explained by an observation on an experimental chimpanzee colony which suggests that colds may be infectious during the incubation period before the onset of any clinical symptoms or signs of the disease.

It is not necessary to assume that all colds, although they may be very similar in their character and sequelae, are caused by the same agent. The initial symptoms of measles and a number of other diseases are very similar to those of the common cold; experimental spraying of the upper respiratory tract with pure cultures of the Pfeiffer bacillus and of some other bacteria has, in several instances, produced symptoms of the common cold. At the same time painstaking examination of the bacterial flora of the mouth and nose extending over considerable periods has shown that there is no significant increase in number or change in type of bacterial flora coincident with the development of a cold in the individual under observation. Furthermore, inoculation of volunteers with strains of a variety of organisms isolated from patients with colds has given no evidence of a consistent causal relationship. Many investigators have hence been driven to search for a more specific cause with attention being directed toward filterable agents.

The Virus. Observations by Kruse[58] in 1914, by Foster[59] in 1916, and later by others, gave indications of the presence of a filterable virus in colds, but several subsequent investigators failed to get similar results. Two later investigators[60,61] indicated that chimpanzees are susceptible to colds which are practically identical with colds observed in man, that chimpanzees and human beings given intranasal instillations of bacteria-free filtrates of nasal washings from early human cases of colds develop an acute respiratory infection indistinguishable from the natural common cold, and that this disease can be transmitted through a series of human volunteers or experimental animals by successive inoculations of bacteria-free filtrates from nasal washings. There was some evidence that the agent was cultivable upon chick embryo medium. More recently other reports have appeared giving evidence for the cultivability of the common cold virus in embryonated eggs.[62,63]

Prophylaxis. Control of the common cold is an even more difficult task than that of most droplet-borne infections because of the well-nigh universal distribution of the virus. Favorably situated individuals may perhaps do something to minimize contacts, but the average active person in an urban community is likely to be rather frequently exposed.

Recovery from a cold apparently is accompanied by a transient immunity, if any; some persons can experience several attacks within a period of a few months. Attempts to immunize man with active virus have been unsuccessful,

---

[58] Kruse: Münch. med. Wchnschr., 1914, 61:1547.

[59] Foster: Jour. Amer. Med. Assn., 1916, 66:1180; Jour. Inf. Dis., 1917, 21:451.

[60] Dochez, Mills and Kneeland: Jour. Exp. Med., 1936, 63:559: Dochez: Jour. Amer. Med. Assn., 1938, 110:177.

[61] Long, et al.: Jour. Exp. Med., 1931, 53:447.

[62] Pollard and Caplovitz: Science, 1947, 106:243; Pollard: Amer. Jour. Hyg., 1948, 47:103, 106.

[63] Topping and Atlas: Science, 1947, 106:636.

but various "cold vaccines," consisting of killed bacterial emulsions, are on the market. Their efficacy in preventing colds is questionable, although some may be of value in preventing infection by secondary invaders, following primary infection of the cold virus.

MEASLES (RUBEOLA)[64]

Although measles is one of the commonest diseases of childhood, it readily attacks adults in populations not previously exposed, or in groups in which an appreciable number of susceptibles are present. During World War I it was prevalent in the Army camps of the United States but it was not a problem during World War II. There is a tendency to look upon measles as one of the
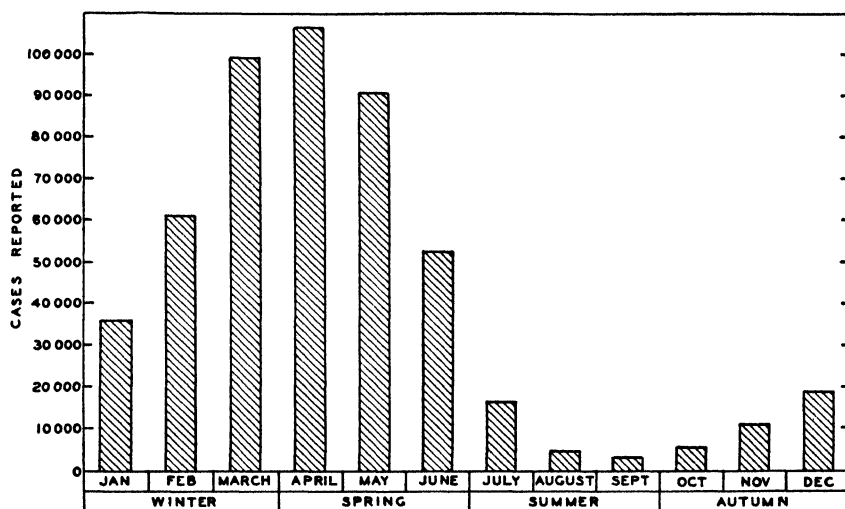
Fig. 246. The seasonal incidence of measles. Averages of reported cases by months for the years 1939 to 1945 inclusive. Data from Supplements to Public Health Reports.

harmless diseases of childhood because of its general prevalence. Although it has a low case fatality rate, over 1200 deaths were recorded in the Registration Area during 1942. The median number of cases reported in the United States in recent years is 612,000 per year.

The disease is characterized in the prodromal stage by an upper respiratory infection with conjunctivitis, catarrh and fever. Later the characteristic enanthem (Koplik spots) in the buccal mucosa and the exanthem of the skin appear. Although not dangerous when uncomplicated, measles may be followed by streptococcal or pneumococcal pneumonia or other secondary infections. The virus is present in nasopharyngeal secretions and a case is infectious in the pre-eruptive stage. Infectivity is high and almost all exposed children develop the disease.

The Virus. Hektoen[65] found that blood drawn from the veins of a patient and mixed with ascitic broth gave no visible growth when incubated at 37°

[64] Cf. *Virus and Rickettsial Diseases.* Harvard School of Public Health Symposium. Harvard University Press, Cambridge. 1940, p. 237.

[65] Hektoen: Jour. Inf. Dis., 1905, 2:238.

C. for twenty-four hours. But a few milliliters of this mixture injected into a healthy man produced the typical symptoms and eruption after the usual period of incubation. Sellards,[66] however, in carefully controlled observations upon eight subjects failed to transmit measles by the injection of blood.

Anderson and Goldberger[67] seemingly succeeded in communicating the disease to the lower animals (monkeys) in definite and regular fashion. The apparent reason for the preponderating negative and irregular results of earlier experimenters is the fact that human blood is infective for monkeys during a very limited period. This period begins before the appearance of the characteristic eruption and continues only a limited time thereafter.

Further experiments by the same investigators demonstrated the presence of the virus of measles in the mixed buccal and nasal secretions, and showed that the virus could pass through a Berkefeld filter, could resist desiccation for twenty-five and a half hours and freezing for twenty-five hours, while infectivity of the virus was destroyed by heating for fifteen minutes at 55° C.

Subsequently other investigators confirmed the susceptibility of monkeys and the presence of virus in blood and throat washings.

Several attempts have been made to culture the virus on fertile eggs. Rake and Schaffer[68] reported the passage of the virus through more than twenty serial egg passages, starting with blood or nasopharyngeal washings. The agent apparently multiplies on the chorioallantoic membrane but produces no constant lesion to denote its presence. Inoculation of monkeys with the culture virus is followed by one or more of the signs of measles in man—fever, coryza, leucopenia, Koplik spots and exanthem.

During the course of the egg cultures these investigators found further evidence that the agent belongs to the group of filterable viruses. It passed through a Seitz EK filter, and withstood treatment with ether sufficient to eliminate bacterial contamination. It can also be stored at the temperature of dry ice.

Immunity. One attack of measles confers immunity; second attacks are very rare. Normal or convalescent serum, or concentrated plasma globulin, is of value in prophylaxis after exposure. Except in very young children, just sufficient antibody should be given to allow a mild infection to occur, which will induce an active immunity. Larger amounts of antibody given as late as the catarrhal stage are able to modify the severity of the attack.

Rake and his colleagues found that the measles virus, after passage in eggs, gave a much modified, mild disease in children. Inoculation with the egg-passage virus apparently conferred appreciable resistance against the disease, whether the exposure was by chance contact with natural cases or by actual inoculation with blood from known cases.

Rubella (German Measles). This relatively mild febrile exanthema of childhood is usually regarded as a viral disease, in the absence of any evidence for a cultivable or visible etiologic agent. Although of no serious significance as a disease of children, rubella in pregnant women has been found to be associated with congenital malformation in the infant, principally cataract,

[66] Sellards: Bull. Johns Hopkins Hosp., 1919, 30:257.
[67] Anderson and Goldberger: Pub. Health Repts., 1911, 26:847, 887.
[68] Rake and Schaffer: Jour. Immunol., 1940, 38:177.

deafness and cardiac defects.[69] It appears that when the attack occurs during the first two months of pregnancy it is almost certain that the child will be malformed. In the third and fourth months the incidence of malformation drops off sharply. The fetal defects are presumed to be caused by transplacental passage of the virus and actual infection of the embryo.

MUMPS

This highly contagious disease of man, often contracted in childhood, usually takes the form of a swelling of the parotid gland, and is sometimes called *epidemic parotitis*. This gland is not the only vulnerable organ, however; the submaxillary and sublingual glands, testis, ovary, pancreas, and perhaps other glands are attacked, either accompanying or in the absence of parotid involvement. Patients may also exhibit signs of encephalic disturbance, which may be very mild and manifested only by pleocytosis of the spinal fluid, or may be more severe.[70] Inflammation and atrophy of various cranial nerves have also been seen associated with mumps. As in the case of measles, convalescent human serum may be used for prophylaxis after known exposure and during the incubation period, which averages about eighteen days. Approximately 196,000 cases have been reported each year in the United States recently.

A number of experimenters have produced pathological changes in small animals by the use of material from cases of mumps, but because of inability to pass the infection indefinitely in animals, or for other reasons, most of these reports do not convince the reader that the etiological agent of mumps was demonstrated. Johnson and Goodpasture,[71] however, submitted convincing evidence that a filterable agent present in the saliva of early cases of mumps, when inoculated into Stensen's duct of monkeys, causes experimental mumps, an observation that has been confirmed repeatedly. The virus has been adapted to the embryonated egg,[72] and like influenza virus has the ability to agglutinate erythrocytes of the fowl and other species.

A complement-fixation test has been developed[73] in which infected monkey parotid gland, or egg fluid, serves as antigen. Positive sera are found in most persons with a history of mumps, and in many without, indicating the prevalence of unrecognized infection. A similar antigen used in a skin test[74] indicates the immunologic state of the individual.

Investigations are now under way on the use of formalinized or otherwise modified virus for active immunization.[75]

THE PSITTACOSIS-LYMPHOGRANULOMA GROUP

It has been recognized that a number of viruses, whose relationship was not formerly suspected, are in fact similar, and constitute a more or less definite

[69] *Cf.* Potter: Amer. Jour. Pub. Health, 1946, 36:101.

[70] Holden, Eagles and Stevens: Jour. Amer. Med. Assn., 1946, *131*:382.

[71] Johnson and Goodpasture: Jour. Exp. Med., 1934, *59*:1; Amer. Jour. Hyg., 1935, 21:46.

[72] Habel: Pub. Health Repts., 1945, 60:201.

[73] Enders, Cohen and Kane: Jour. Exp. Med., 1945, 81:119.

[74] Enders, Kane, and Maris: Jour. Exp. Med., 1946, 84:341.

[75] Stokes, *et al.*: Jour. Exp. Med., 1946, 84:407.

group[76] on the basis of morphologic, antigenic and certain other relationships. Included within this group are several animal strains as well as the following viruses affecting man; psittacosis, ornithosis, certain strains from human pneumonitis, lymphogranuloma venereum, and less definitely related, trachoma and inclusion conjunctivitis.

These agents all have in common the presence of a characteristic coccoid elementary body demonstrable in smears of infected tissues (Fig. 247), and all the evidence indicates that these are the infective particles of the virus. They may be stained by Giemsa, Castaneda's rickettsial stain, or by Machiavello's method.* It has been shown with several of these viruses, and is doubtless true of all, that the elementary body is one stage in a developmental cycle which is described (p. 879) in the discussion of psittacosis virus.

All the members of this group are infective for mice by the intranasal route, producing a pneumonitis, and many are also pathogenic by the intracerebral or intraperitoneal route. They have some morphological resemblance to the rickettsiae and, like these agents, multiply to a high titer in the yolk sac of fertile eggs. Antigenic similarities have been described between various members of the group on the basis of the complement-fixation test, a test which apparently depends upon an antigen distributed throughout the group. Antigenic differences are demonstrable by cross immunization experiments and by a neutralization test using immune rooster serum.[77]

In contrast to other viruses, many of this group have been shown to be susceptible to the sulfonamides and to penicillin and other drugs.[78] This susceptibility to chemotherapeutic agents suggests that the viruses of this group possibly have some degree of independent metabolism, and indicates that they are more closely related to bacteria than are other viruses. This similarity to bacteria is brought out also by other evidence such as the lack of homogeneity of the infectious particle, as revealed by electron micrographs (Figs. 248, 251).

Psittacosis.[79] This disease, known during the past sixty-five years, was brought into special prominence by the large number of cases appearing in Argentina, Europe and North America in 1929–30. The disease appears in persons associated with birds, and manifests itself in the form of small outbreaks confined to members of families or to people living or working in the same location. The connection with birds is always striking and transmission to man seems to be almost invariably from infected birds. Occasional instances

---

* The last named, because of the marked contrast between color of elementary bodies and tissue elements, is the most satisfactory for general purposes. The method is as follows:

   a. Flood the dried slide with 0.25 per cent basic fuchsin freshly made up in phosphate buffer of pH 7.4. Leave for five minutes.

   b. Destain five to ten seconds with 1 per cent citric acid and wash quickly with tap water.

   c. Stain ten seconds with 1 per cent aqueous methylene blue.

The elementary bodies stain with carbol fuchsin and retain the color in the presence of weak acid. Tissue elements are destained by this method except in the thicker portions of the smear. Destaining should progress only to the point where the thinner portions of the smear are pinkish-grey in color.

76 Beck, Eaton and O'Donnell: Jour. Exp. Med., 1944, 79:65.

77 Hilleman: Jour. Inf. Dis., 1945, 76:96.

78 Smadel and Jackson: Proc. Soc. Exp. Biol. Med., 1948, 67:478.

79 Cox: Ann. New York Acad. Sci., 1947, 48:393.

of apparent transmission from one human being to another have, however, been observed.

Although a number of species of birds are susceptible, infection of man has been brought about almost entirely by contact with parrots or parakeets, including Australian budgerigars. Meyer, recognizing that other than psittacine birds are affected with this type of virus and can transmit the infection to man, has proposed the term "ornithosis."[80] At the present time both terms are in use, psittacosis retaining its former connotation and ornithosis (p. 881) indicating infection with similar viruses from non-psittacine birds.

The Avian Disease. Infection with the virus of psittacosis appears to be enzootic among psittacine birds in the natural state, at least in Australia, and
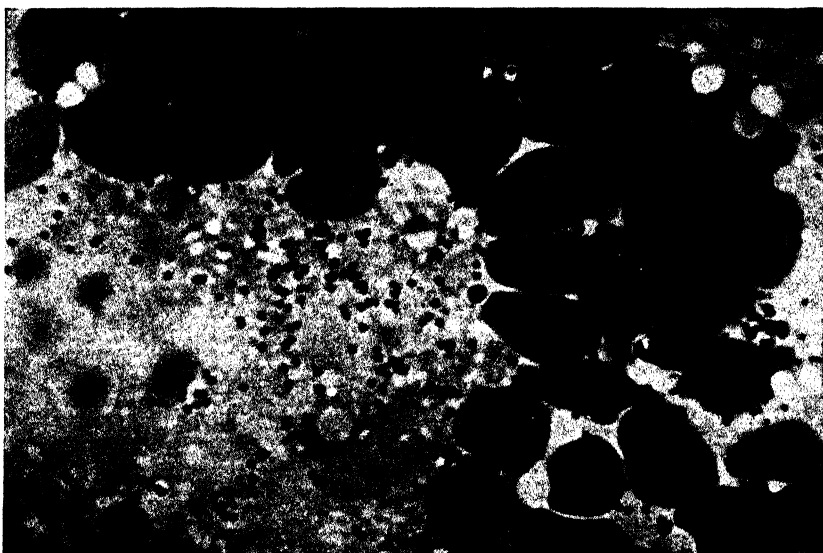
Fig. 247. Elementary bodies of psittacosis. Impression preparation from infected mouse spleen; reduced from × 2575. (Obtained through the courtesy of S. P. Bedson.)

the disease has been discovered in birds imported from several countries. Birds in the acute stage of the infection show emaciation, weakness and ruffling of the feathers. A nasal discharge and the watery cloacal excretion both may contain the virus. The conspicuous pathologic lesion is a focal necrosis of the liver. The incubation period may be very long and recovery may result in a prolonged carrier stage. Healthy-appearing carriers of the virus make it difficult to prevent importation of infected stock.

The Human Disease. It is assumed that the portal of entry in man is the respiratory tract. Contact with infected birds is unnecessary, indirect contact through fomites, or even proximity to infected material, being sufficient to cause infection in man. The degree of infectivity, approaching that of measles and smallpox, as indicated in a study by McCoy, has made laboratory investigation of this disease particularly difficult.[81] The infection in man is often

[80] Meyer: Medicine, 1942, 21:175.
[81] McCoy: Pub. Health Repts., 1930, 45:843.

severe, and a rather high case-fatality rate has been the rule. It usually has an acute onset and takes the form of a pneumonia, with, however, a paucity of sputum.

Active immunization of laboratory workers was first accomplished with active virus given intramuscularly. Formalin-inactivated virus has been shown to confer resistance upon laboratory animals.[82]

The Virus. For some time after 1893, when Nocard isolated a gram-negative bacterium from the bone marrow of parrots which had died with psittacosis, it was thought that the causative agent had been found. Subsequent work showed that this organism, which turned out to be *Bacterium typhi-murium*, was not of etiological significance. During the 1930 outbreak several groups of investigators demonstrated the filterable nature of the agent.
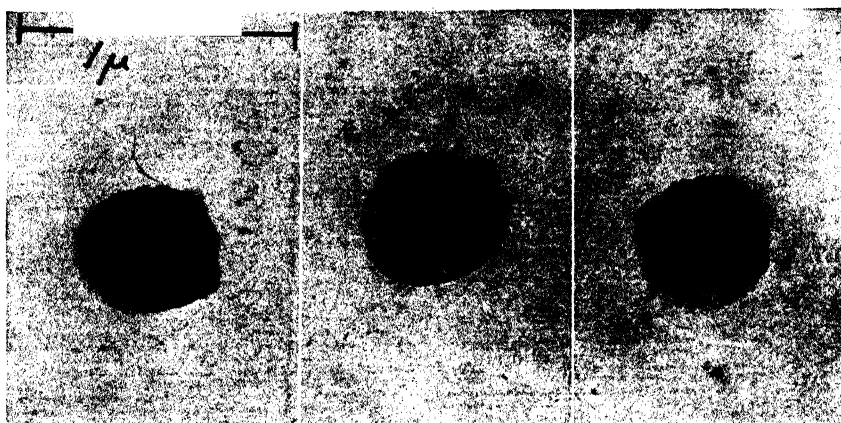
Fig. 248. Electron micrographs of the elementary bodies of the psittacosis virus. (SAB No. 200.)

The virus is present in the blood and sputum of patients with the disease and in the tissues of infected animals. After intraperitoneal inoculation of a mouse, the animal used mainly for investigation of this disease, virus is present in the spleen, and intranasal inoculation produces a pneumonitis. Intradermal injection of virus into the skin of rabbits and guinea pigs produces a papular eruption similar to that caused by herpes virus. A pneumonia not unlike that seen in man follows intratracheal inoculation of monkeys, and a meningo-encephalitis develops after intracerebral injection of mice, monkeys, rabbits and guinea pigs.[83] Cultivation in tissue culture and on the chorioallantoic membrane of eggs is successful,[84] but a more susceptible tissue is the yolk sac.

Examination of infected material shows the presence of small coccoid bodies, arranged singly and in pairs, illustrated in Fig. 247. They can be stained by the methods indicated on p. 845. Centrifugal sedimentation and serological reactions furnish evidence that these bodies are the virus. Levinthal first called

[82] Wagner et al.: Jour. Immunol., 1946, 54:35.

[83] Rivers, Berry and Sprunt: Jour. Exp. Med., 1931, 54:91; Rivers and Berry: *ibid.*, 105, 119, 129.

[84] MacCallum: Brit. Jour. Exp. Path., 1936, 17:472; Burnet and Rountree: Jour. Path. Bact., 1935, 40:471.

attention to considerable variation in size and form of these structures and gave them the name *Microbacterium multiforme psittacosis*. Lillie proposed the name *Rickettsia psittaci*, but this has not been accepted because no arthropod host has been demonstrated. They are often called elementary bodies of psittacosis and, because they were described at about the same time by the two workers mentioned above and by Coles, they are also known as L.C.L. bodies.

Developmental Cycle. A number of investigators have studied the morphology of this virus and have found that the elementary bodies represent only one stage in a developmental cycle. The descriptions by various observers,[85] although differing in minor respects, are in general agreement and may be summarized as follows:

The elementary body, estimated at 300 to 450 mμ in diameter, when placed in a susceptible tissue increases in size to become an "initial body" with a
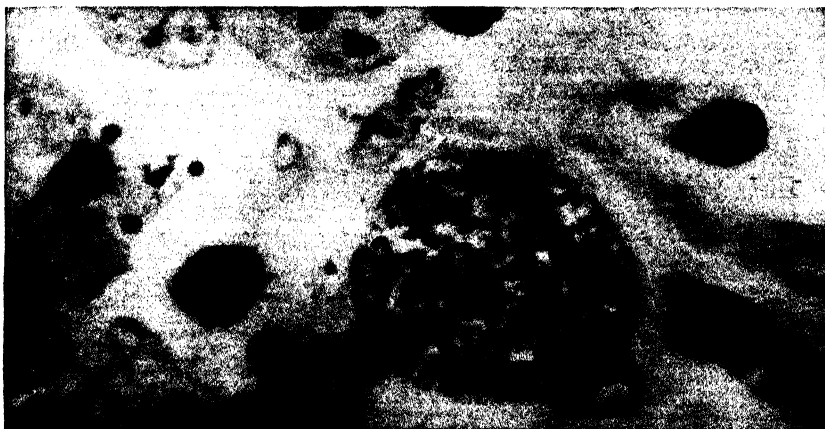
Fig. 249. A vesicle containing elementary bodies from the lung of a mouse infected with mouse pneumonitis. This is similar to the final stage in the developmental cycle of psittacosis virus. Noble's stain (see ref. 85); × 2100.

diameter two to four times that of an elementary body. With multiplication by fission (according to most observers) and the development of a matrix, which may be produced by the virus or by the host cell, a "plaque" is formed which may be several microns in diameter. With many stains this appears to be a homogeneous structure, but proper manipulations reveal corpuscular elements in the process of division within the matrix. As development proceeds the matrix material becomes less dense, revealing particles which become of smaller average size during continued division until eventually a large vesicle (Fig. 249) is formed consisting of a mass of elementary bodies. This vesicle eventually ruptures, releasing elementary bodies for the infection of other cells. The whole cycle requires forty-eight to seventy-two hours. Histopathologic studies by Weiss[86] on three other viruses of this group (mouse pneumonitis, feline pneumonitis and meningopneumonitis) confirm these observations, but also

[85] See the summary of previous work and observations recorded by Yanamura and Meyer: Jour. Infec. Dis., 1941, 68:1.
[86] Weiss: Jour. Infec. Dis., 1949, 84:125.
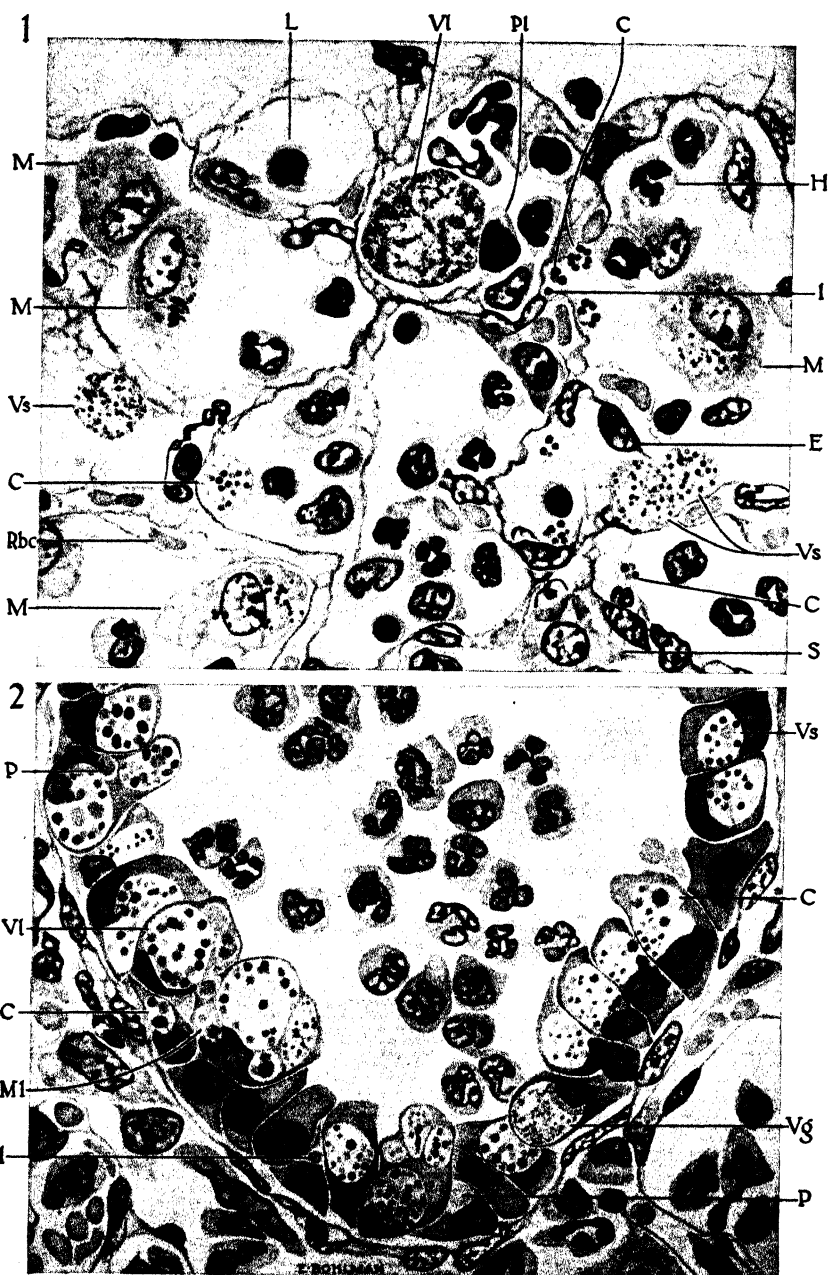
Fig. 250. Mouse pneumonitis virus in mouse lung. Hematoxylin-eosin-azure II. Reduced from a magnification of × 1600. Section 1. Extracellular virus in the alveoli. The mouse was vitally stained with trypan blue before inoculation with the virus. Section 2. Intracellular virus in the epithelium of a bronchiole. C, cluster; E, endothelial cell; H, heterophile; I, initial body; L, lymphocyte; M, macrophage (note trypan blue pigment in the cytoplasm); MI, multiple infection of single cell; P, plaque; Rbc, red blood cell; S, septal cell; Vg, finely granular vesicle; Vl, large vesicle; Vs, small vesicle. (Weiss, Journal of Infectious Diseases, 1949.)

offer convincing evidence that the cycle occurs extracellularly in the alveolus of the mouse lung. (Fig. 250.) In other tissues, development is intracellular. An interesting observation recorded by two different observers (and also described as occurring in the similar cycle of lymphogranuloma venereum virus) is that the cycle is different if the infective psittacosis particle enters a cell already damaged. In this case no plaque is formed; the elementary bodies multiply by continued fission retaining the general size and character of elementary bodies until the vesicle stage is reached.

Laboratory Diagnosis. Rivers and Berry[87] described a rapid diagnostic method for the human disease which consists of inoculating mice intraperitoneally with filtered or unfiltered sputum. Specific infection in the mice may be recognized by typical lesions and the presence of L.C.L. bodies in spleen and liver, or by subsequent immunity to inoculation with a known strain of psittacosis virus. The high susceptibility of the mouse brain also makes this tissue valuable in detecting the virus. Complement-fixation tests, also of value in the diagnosis of human infections, have been found positive as early as the twelfth day of the disease. This test has been used also in the recognition of latent infection in psittacines imported into this country.

Ornithosis. A number of strains of psittacosis-like virus have been isolated from birds other than parrots and parakeets, and the term ornithosis has come to be used to distinguish these from true psittacosis strains. A disease occurring in the Faroe Islands and traced to contact with fulmar petrels in 1938 yielded a virus similar to psittacosis, and this was one of the first indications that viruses of this type occurred in other species than those of the Psittacidae. On numerous occasions pigeons have been shown to be infected, and atypical pneumonia in man has been traced to contact with pigeons. Domestic fowl also can harbor ornithosis virus and presumably transmit the infection to man. Various domestic birds of Michigan have yielded sera capable of fixing complement with meningopneumonitis antigen, thereby indicating infection with a virus of this group. Similar strains have been isolated from cases of atypical pneumonia in man.

Representative strains of ornithosis virus differ slightly from psittacosis strains in pathogenicity for experimental animals, and Hilleman[77] has found that they are antigenically quite different as determined by a neutralization test.

Meningopneumonitis. This virus was originally isolated in ferrets after inoculation with human throat washings[88] and was named for the characteristic pathology produced in inoculated mice and other experimental animals. Although not obviously associated with birds it resembles ornithosis virus very closely and considerable evidence indicates that it is identical with that virus.

Human Pneumonitis. Several strains of virus isolated by Eaton and his colleagues (*e.g.* strain SF) from man can be distinguished from the other members of the group on various bases. They appear to be most closely related to psittacosis strains but a difference in pathogenicity for the mouse when intraperitoneal inoculation is used has been described.

[87] Rivers and Berry: Jour. Exp. Med., 1935, *61*:205.
[88] Francis and Magill: Jour. Exp. Med., 1938, 68:147.

Strains isolated in Louisiana[89] from a severe outbreak of pneumonitis in man were recognized as belonging to this group. The strains (*e.g.*, *Borg*) are distinguishable antigenically and because of their great virulence and wider host range. The epidemic was characterized by high infectivity for contacts, and absence of any association with birds.

Strains isolated in Chicago[90] from fatal cases of human pneumonitis have been called *Illinois* virus. This virus also is antigenically different from others of the group.

Lymphogranuloma Venereum.[91] Although known during the nineteenth century, this venereal disease, also called *lymphopathia venereum*, *lymphogranuloma inguinale** and *climatic bubo*, has come into prominence only during the present century, and there is much evidence that it has increased in incidence in many parts of the world.[92] At first believed to be confined to the tropics and subtropics, it has since been recognized not only in seaports of the temperate regions but in inland cities as well. It has been found in most regions of the United States but is especially prevalent in the South, where the incidence is high in the Negro race.

The primary lesion, occurring upon the genital organs, is usually small and is often overlooked by the patient and not brought to a physician's attention. Extension into the lymphatics produces the typical manifestation of inguinal bubo in the male, while in the female, lymph nodes within the pelvis are involved. The chronic inflammation in this locality may result in rectal stricture; extensive ulceration in the perineal region and hypertrophic lesions of the external genitalia may also occur.

The Virus. Successful transmission to animals was first accomplished by Hellerström and Wassén (1930) who found that monkeys proved susceptible to intracerebral inoculation of pus from inguinal buboes. Since then mice also have been found susceptible. Isolation of the virus has been accomplished by injection of the brains of mice or the yolk sacs of fertile eggs with the contents of a bubo. Monkeys and mice react to intracranial injections with a meningoencephalitis, and intradermal or subcutaneous inoculation in several species is reported to result in papular lesions in which the virus may be demonstrated. Propagation on several types of tissue culture has been successful and, like other members of this group, the virus reaches a high titer when grown in the yolk sac of fertile eggs.

The elementary bodies of this virus were first known as the granulocorpuscles of Miyagawa, but it was not until some time later that all the various morphologic elements associated with this infection were described. The developmental cycle has been observed by several investigators, but is best illustrated in a paper by Rake and Jones[93] who also summarize previous reports. The cycle is strikingly similar to that observed for psittacosis virus and described on page 879. Rake and his colleagues have described a soluble antigen

* Not to be confused with *granuloma inguinale* (p. 554).

[89] Olson and Treuting: Pub. Health Repts., 1944, *59*:1299; Olson and Larson: Pub. Health Repts., 1945, *60*:1488.

[90] Zichis and Shaughnessy: Science, 1945, *102*:301.

[91] *Cf.* p. 365 of *Virus and Rickettsial Diseases.* (Ref. 64.)

[92] *Cf.* the review by D'Aunoy and von Haam: Arch. Path., 1939, *27*:1032.

[93] Rake and Jones: Jour. Exp. Med., 1942, *75*:323.

associated with this virus, which can be demonstrated by the complement-fixation test, and from the same source have come reports[94] of a toxin in emulsions of this virus as well as the related ones, meningopneumonitis, mouse pneumonitis and feline pneumonitis. The toxins have the characteristics of labile endotoxins and antitoxins prepared against them are highly specific.

Immunity. Frei[95] found that intracutaneous administration of heated pus from an infected bubo results in an inflammatory necrotic area at the site of inoculation. The material also induces a febrile reaction when given intravenously. Brains of infected mice have also been used as a source of Frei antigen but at the present time a commercial preparation of infected yolk sac emulsion, marketed under the name "Lygranum," is commonly used.

Complement-fixation tests, using the patient's serum, have been used extensively for diagnosis, and the infected yolk sac antigen is used for this purpose also. A limitation of the test is the fact that the antigen involved is found
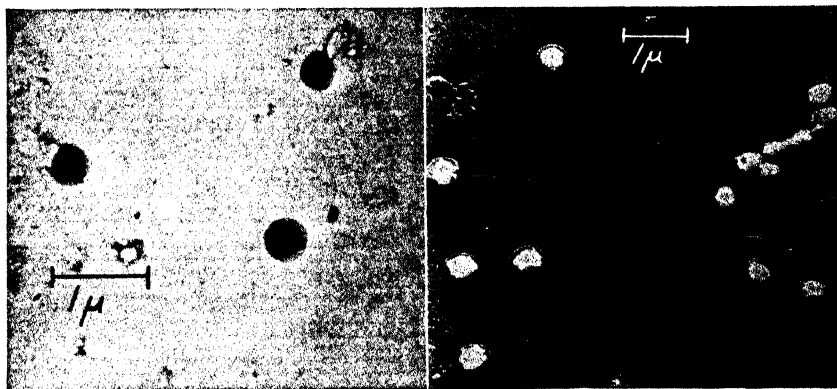
Fig. 251. Electron micrographs of the elementary bodies of feline pneumonitis virus. Left, the usual preparation; right, shadow-cast preparation. (SAB No. 192.)

in other members of the group. Thus, it has been demonstrated that psittacosis convalescent serum readily fixes complement in the presence of the lymphogranuloma antigen.

Mouse Pneumonitis. A virus latent in certain mouse stocks has been encountered by several investigators.[96] Continued intranasal passage of lung tissue in such stocks results in an increase in virulence so that the mice die with extensive consolidation of the lungs. Practically, it is a potential hazard in working with other pneumotropic viruses in mice because of the possibility of contamination of the other strain from carrier mice. When using mice for work with other pneumotropic agents one should exert every effort to provide himself with mice free from this virus.

Feline Pneumonitis. In 1942 Baker[97] described the isolation from sick cats of a virus infective intranasally for mice and other animals and for the yolk sac of fertile eggs. The virus, associated with elementary bodies similar to

[94] Rake and Jones: Jour. Exp. Med., 1944, 79:463.
[95] Frei: Klin. Wchnschr., 1925, 4:2148.
[96] Nigg and Eaton: Jour. Exp. Med., 1944, 79:497; Karr: Jour. Inf. Dis., 1943, 72:108.
[97] *Cf.* Baker: Jour. Exp. Med., 1944, 79:159.

the others of this group, is present in the respiratory tract as well as the ocular and nasal discharges of the cats. Transmission was obtained by contact.

Ann Arbor Virus.[98] Of uncertain origin is this strain isolated in a test of human throat washings. It has affinities with a mouse pneumonitis strain but is not antigenically identical.

TRACHOMA

Trachoma is a chronic disease of the eye characterized primarily by papillary or follicular hypertrophy of the conjunctiva, with vascularization, or "pannus" formation, in the cornea and secondarily, by cicatrization of the conjunctiva and destructive changes in the lids and cornea. In some cases it produces partial or total blindness. Often, in the early stages and at intervals during its course, there is acute inflammation accompanied by secondary bacterial infection which is no doubt partly responsible for the inflammatory condition. Although trachoma can be transmitted by contact, ordinary hygienic precautions are sufficient to prevent infection of those in daily association with patients. Affecting a large proportion of the population in some countries, trachoma in the United States is largely confined to some sections of Kentucky, Tennessee, Illinois and Missouri, and to some of the Indian reservations.[99]

Cell inclusions in the conjunctiva are regularly encountered in the human infection, being present with sufficient frequency to provide a simple means of differentiation of early cases from follicular conjunctivitis and other clinically similar conditions. These inclusion bodies have long been regarded as representing the etiological agent of the disease. Halberstaedter and von Prowazek,[100] who first described the cell inclusion, believed it to be a Chlamydozoon and classed the disease with smallpox. Later a number of workers came to the belief that these bodies represent "nests" of growing bacteria or cell-reaction products stimulated by bacterial infection.

Hemoglobinophilic bacilli isolated from cases of trachoma were apparently associated with complicating bacterial conjunctivitis. A small, feebly motile, hemoglobinophilic bacillus, *Bacterium granulosis*, isolated by Noguchi,[101] was the subject of extensive investigations and for a time was widely accepted as the cause of the disease, although general confirmation of the findings of Noguchi and others was lacking.

Since 1930 the chief emphasis in investigation has shifted again to the inclusion body. Transmission of trachoma to monkeys with infective material free of all bacteria has been accomplished with filtrates[102] and with material from testicular passage[103] in animals. In spite of extensive search the characteristic inclusion body has not been found in the experimental disease. It has

[98] Kempf, Wheeler and Nungester: Jour. Inf. Dis., 1945, 76:135.

[99] For a thorough discussion of trachoma, including its history, epidemiology and etiology, see Julianelle: *The Etiology of Trachoma*. The Commonwealth Fund, New York. 1938.

[100] Halberstaedter and von Prowazek: Deut. med. Wchnschr., 1907, 33:1285.

[101] Noguchi: Bull. N. Y. Acad. Med., 2nd Ser., 1927, 3:295; Jour. Exp. Med., 1928, 48: Suppl. 2.

[102] Thygeson: Amer. Jour. Ophth., 1933, 16:409; Thygeson and Proctor: Arch. Ophth., 1935, 13:1018; Julianelle and Harrison: Amer. Jour. Ophth., 1935, 18:133.

[103] Julianelle and Harrison: Amer. Jour. Ophth., 1937, 20:354.

been emphasized that the elementary bodies of the cell inclusion in man are closely identified with the virus of trachoma. This relationship appears to be even more clearly established from inclusion conjunctivitis, an eye infection of the newborn clinically similar to trachoma and with inclusions which are morphologically like those of trachoma.

The similarity of these inclusions to those of other viruses, especially psittacosis and lymphogranuloma venereum, has been pointed out.[104] The large inclusions of trachoma and inclusion conjunctivitis give a positive test for glycogen while those of the latter agents do not; otherwise they are morphologically quite similar. It has also been reported[105] that sera of patients with these two eye diseases will fix complement with antigen prepared with the virus of lymphogranuloma venereum, indicating relationship with the psittacosis-lymphogranuloma group.

Claims that the virus of trachoma is rickettsial[106] are not surprising when one remembers the morphologic similarity between rickettsiae and the elementary bodies of psittacosis and related viruses, to which group trachoma has a claim. However, the report that rickettsiae appeared in the intestines of lice after inoculation with trachomatous material and that material from the passage lice was infective could not be confirmed.[107]

Favorable results in the treatment of trachoma and inclusion conjunctivitis with sulfonamide drugs have been reported,[108] but there is incomplete agreement as to whether this is due to an action of the drug upon the virus or upon the secondary bacterial invaders. If the former is true it constitutes another relationship between these viruses and those of the psittacosis lymphogranuloma group, which are susceptible to the sulfonamides.

LYMPHOCYTIC CHORIOMENINGITIS

Lymphocytic choriomeningitis is the name given to a specific infection which in man has been included under the general clinical terms *acute aseptic meningitis,*[109] *idiopathic meningitis,* etc. Apparently only a portion of these cases, defined by clinical characteristics, are due to the virus of lymphocytic choriomeningitis,[110] and indeed the latter diagnosis is not justified unless the virus is isolated from the patient, or the development of specific antibody is demonstrated in the serum.

Human Infection. The disease in man begins with a mild upper respiratory infection and some cases do not progress beyond this stage, running their course as an influenza-like illness. In typical cases, a few days after the onset of the prodromal signs, symptoms referable to the nervous system make their

[104] *Cf.* Rake and Jones: Jour. Exp. Med., 1942, 75:323.
[105] Rake, Schaffer and Thygeson: Proc. Soc. Exp. Biol. Med., 1942, 49:545.
[106] Busacca: Folia clin. biol. São Paulo, 1933, 5:96; Arch. Ophth., Paris, 1935, 52:567. Cuenod: Arch. Inst. Pasteur, Tunis, 1938, 24:86. Rev. internat. trachome, 1936, 13:9. Cuenod and Nataf: Arch. Inst. Pasteur, Tunis, 1936, 26:295; Rev. internat. trachome, 1937, 14:104; Brit. Jour. Ophth., 1937, 21:309.
[107] Weigl: Centralbl. f. Bakt., I Abt., Orig., 1939, 143:291; Braley: Arch. Ophth., 1939, 22:262.
[108] Thygeson and Stone: Jour. Amer. Med. Assn., 1942, 119:407.
[109] Wallgren: Acta paediat., 1925, 4:158.
[110] Baird and Rivers: Amer. Jour. Pub. Health, 1938, 28:47.

appearance. These may consist of sudden severe headache with stiffness of the neck, fever, vomiting, drowsiness, or more severe nervous-system signs. The spinal fluid shows an increased number of cells, most of which are mononuclear. Complications are infrequent and complete recovery is the rule. The virus is present in the blood and spinal fluid during the early stages and has also been demonstrated in the nasopharynx. Serum antibodies make their appearance during convalescence. In a survey[111] in which 1248 human sera were tested, 11 per cent showed neutralizing ability. Only 32 per cent of sera from cases diagnosed as aseptic meningitis neutralized the virus. Neutralization tests are positive some weeks after recovery, but positive complement-fixation reactions, dependent upon the presence of a soluble antigen associated with the virus, appear earlier and may prove to be of more value in diagnosis of the disease.

Animal Reservoirs. This virus was encountered by Armstrong and Lillie in 1934 as a contaminant in monkeys inoculated with the virus of St. Louis encephalitis, and it was the pathological picture in monkeys which gave this infection its name. It was also found to be identical with the agent of a spontaneous disease of mice, described by Traub in 1935,[112] which is transmitted from mother to young in the mouse colony and is maintained by healthy carriers. The virus has since been isolated from human cases,[113] and has been encountered in other monkey and mouse stocks. It has also been found in wild mice trapped in households in which human cases occurred, suggesting that the association of human and murine infection is of etiological significance.[114]

The Virus. The virus is pathogenic for mice, monkeys and guinea pigs, some investigators finding the latter two species more suitable for primary isolation from man. Inasmuch as this virus occurs naturally in several species of laboratory animal, isolation of the virus by animal inoculation means little unless the animals are known to be free of the infection. Mice with an acute infection are passive and ruffled, or when disturbed exhibit tremors and convulsions. After inoculation by any of several routes virus may be found in the brain and blood and often in urine and nasal secretions. Guinea pigs are susceptible to intracerebral or subcutaneous injections and show fever and loss of weight but no definite neurological signs. The characteristic lesion in experimental animals, as the name indicates, is present in the meninges and choroid plexuses in the form of an inflammation. Monkeys may be infected by a variety of routes and at death virus is found widely distributed throughout the body.

Modification of typical strains has been reported in which repeated passage in guinea pigs has reduced the virulence for mice, and vice versa. Immunization of guinea pigs with the mouse-adapted strain, and with formolized virus, has been reported. A soluble substance associated with this virus can serve as antigen in the complement-fixation test.[115] Spleen tissue of infected guinea pigs is a good source of antigen.

[111] Armstrong and Wooley: Jour. Amer. Med. Assn., 1937, *109*:410.
[112] Cf. Traub: Jour. Exp. Med., 1936, *63*:533.
[113] Scott and Rivers: Jour. Exp. Med., 1936, *63*:397, 415.
[114] Armstrong, Wallace and Ross: Pub. Health Repts., 1940, *55*:1222; Dalldorf, Jungeblut and Umphlet: Jour. Amer. Med. Assn., 1946, *131*:25.
[115] Smadel and Wall: Jour. Bact., 1941, *41*:421.

The presence of virus in the blood of experimental animals has led to experiments on arthropod transmission of the disease. Coggeshall[116] found that *Aedes aegypti* is able to transmit the infection by first feeding on an infected guinea pig and later upon a normal one. Shaughnessy and Milzer[117] demonstrated similar experimental transmission by the wood tick, *Dermacentor andersoni*, and found that the virus was maintained by the tick through its various developmental stages and from one generation to the next. Guinea pigs were also infected by applying crushed ticks or feces to the scarified skin.

Further experiments[118] showed that bedbugs can take up the virus from infected guinea pigs and transmit the infection to normal guinea pigs while biting. Transmission appears to take place by contamination of the skin of the guinea pig by the bedbug feces which has been shown to contain virus as late as eighty-five days after feeding. Transmission by the bites of monkey lice and of mites was not successful but these arthropods were shown to harbor the virus. Whether any of these findings represent a factor of significance in the natural transmission of this virus is unknown, but it seems likely that arthropods will be found to play some role in its epidemiology.

Trichinella larvae from an infected animal have also been found capable of transferring the infection to another host.[119]

YELLOW FEVER

This disease, at present endemic over large regions in West and Central Africa and in South America, in the past has appeared in epidemic form in seaports of the northern temperate zones on both sides of the Atlantic. Its last appearance in the United States was in 1905 at New Orleans. It is not completely understood why this disease has not spread farther in warm climates, for instance into the Orient, where two important factors for its maintenance are already present, the specific insect vector and a susceptible population. In recent years the facilities for rapid transportation by air and motor highway from the tropics have increased the danger of dissemination, and necessitated strict quarantine measures for travelers from the endemic regions.

The disease when occurring in epidemic form is very severe and carries a high case fatality. The essential damage is to the liver, producing a jaundice in the patient. The lesion, a mid-zonal necrosis in the hepatic lobule (Fig. 252), is pathognomonic of yellow fever. Recovery results in a solid immunity, accompanied by antibody in the serum demonstrable by the serum neutralization test. This antibody apparently persists indefinitely in a recovered patient.

Etiology.[120] Nott in 1848 advanced the hypothesis that insects carried the infection from person to person, and Finlay (1881), convinced that *Aedes aegypti* was the species of mosquito responsible, carried out numerous experiments on human volunteers without convincing results. Carter (1900),[121] in epidemiological studies during an outbreak in Mississippi, defined the limits of the extrinsic incubation period, *i.e.*, the period of time necessary before the

---

[116] Coggeshall: Science, 1939, 89:515.
[117] Shaughnessy and Milzer: Amer. Jour. Pub. Health, 1939, 29:1103.
[118] Milzer: Jour. Inf. Dis., 1942, 70:152.
[119] Syverton, McCoy and Koomen: Jour. Exper. Med., 1947, 85:759.
[120] *Cf.* p. 713 of *Virus and Rickettsial Diseases*. (Ref. 64.)
[121] Carter: New Orleans Med. and Surg. Jour., 1900, 52:617.

"surroundings" of a case became infectious. The period was ten to fifteen days and we now know that this represents the incubation period in the mosquito before it becomes infective. The contributions of Finlay and Carter formed a valuable basis for the experiments undertaken by the American Army Commission (Reed, Carroll, Agramonte, Lazear)[122] in Havana, Cuba, at the beginning of the present century. With the aid of human volunteers the Commission established the following important points:

1. Bacteria previously suspected were not etiologically related to yellow fever.
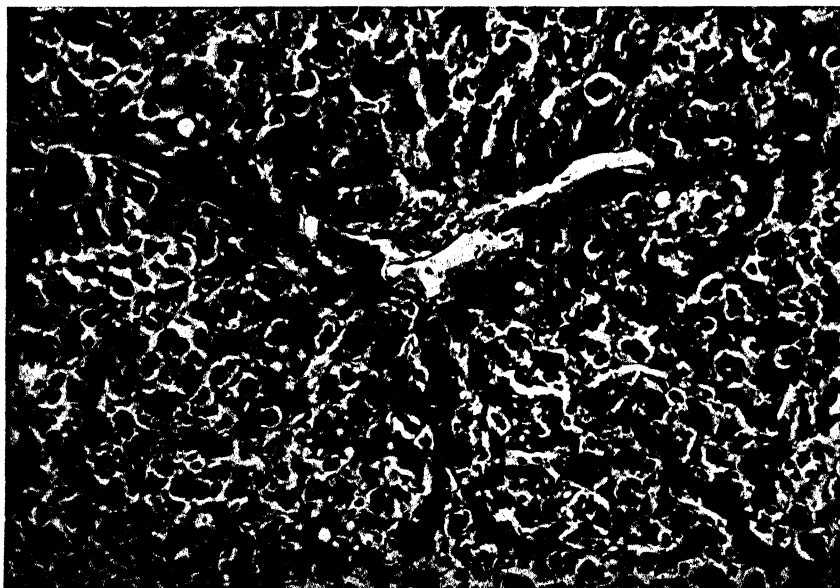
Fig. 252. Section of liver from a human case of yellow fever. Mid-zonal necrosis is apparent. Hematoxylin and eosin; reduced from × 235. (Obtained through the courtesy of Max Theiler.)

2. The living causal agent of this disease was not cultivable nor visible and was filterable through a Berkefeld candle.

3. The malady was transmissible in nature by the bites of female *Aedes aegypti* mosquitoes, and was not communicated to non-immunes through intimate exposure to clothing or bedding contaminated with discharges of patients.

4. A period of about twelve days at summer temperatures was necessary after a mosquito had fed on a patient in the early stage of the disease before it could infect a second individual.

5. The subcutaneous injection of small amounts of blood drawn from a yellow fever patient on the first to third day of the disease was capable of inducing an attack of yellow fever.

[122] Reed, Carroll, Agramonte and Lazear: Proc. 28th Ann. Meeting, Amer. Pub. Health Assn., Oct. 22, 1900; Reed: Med. Record, 1901, *60:*201; Reed and Carroll: Med. Record, 1901, *60:*641; Amer. Med., 1902, 3:301; Carroll: Jour. Amer. Med. Assn., 1903, *41:*1341.

The existence of yellow fever in West Africa, where large areas and populations are affected, has long been regarded as a possible menace to the rest of the world. An even wider distribution of this disease in Africa is now apparent, outbreaks having been reported from Anglo-Egyptian Sudan, Uganda, Eritrea and Northern Rhodesia. These observations together with the results of widespread serum surveys lead to the conclusion that yellow fever is probably endemic throughout most of tropical Africa except the extreme eastern zones.[123] Investigators of the International Health Division of the Rockefeller Foundation began a study of the disease in West Africa in 1925. Monkeys of this area were found to be completely resistant to yellow fever infection, but transmission to Asiatic monkeys (*Macaca mulatta*) was successful, an achievement which made possible an intensive examination into the nature of the disease. Stokes and his colleagues[124] concluded that a filterable virus is the cause of the malady and in general confirmed by means of animal experimentation the work of the commissions of 1900 to 1906. Their experiments were repeated and their results confirmed in West Africa and in South America.[125] Subsequently, yellow fever has been investigated by numerous workers in various countries, and many facts concerning the disease and its agents have been discovered.

The Virus. The virus of yellow fever, one of the smaller viruses, occurs in the serum of infected monkeys in high concentration and passage may be effected using blood or tissues. Emulsions in physiological saline deteriorate rapidly but the presence of serum exerts a marked stabilizing effect. The virus has been propagated in different types of tissue culture and in the embryonated egg.

Neurotropic Virus. A notable advance was made by Theiler[126] when he adapted the virus to mouse brain by intracerebral passage, resulting in loss of virulence for monkeys and man when given subcutaneously, but still producing encephalitis in monkeys if given intracerebrally. Such neurotropic strains maintain their immunological identity with natural, or viscerotropic, strains and are suitable for use in serum neutralization tests. Thus, the use of mice allows much more extensive investigation of various kinds than did the larger more expensive monkey.

Mosquito Transmission.[127] The virus is present in the blood of patients during a few hours preceding the onset and for the first three or four days of illness. A mosquito biting at that time takes up virus with its blood meal. Titrations of the virus content of *Aedes aegypti* have shown that an initial drop occurs followed by an increase. After about twelve days, depending upon the temperature of the environment, virus reaches the salivary glands. The mosquito is then infective and remains so for the rest of its life.

Control of Yellow Fever. The findings of Reed and his colleagues, confirmed by other workers in Cuba and Brazil, suggested measures for combating

[123] Mahaffy, Smithburn and Hughes: Trans. Roy. Soc. Trop. Med. Hyg., 1946, 40:57.

[124] Stokes, Bauer and Hudson: Amer. Jour. Trop. Med., 1928, 8:103.

[125] Aragoa: Jour. Amer. Med. Assn., 1928, 92:550; Davis and Burke: Jour. Exper. Med., 1929, 49:975.

[126] Theiler: Ann. Trop. Med. Parasitol., 1930, 24:249.

[127] *Cf.* p. 735 of *Virus and Rickettsial Diseases.* (Ref. 64.)

yellow fever in the countries then concerned. Gorgas in Havana had previously applied extensive general public health measures and succeeded in markedly reducing the general death rate, but had not influenced the incidence of yellow fever. Following the report of the Army Commission he instituted antimosquito measures directed against *Aedes aegypti*. The result was an immediate, sharp decrease in the incidence of the disease. It was found that destruction of *Aedes aegypti* was necessary only in the large urban centers. These appeared to be the endemic foci of the disease and served to supply virus for outbreaks in other cities and localities in communication with them but sometimes at distances of many hundreds of miles. Such measures were taken in the key cities of many localities in Central and South America by representatives of the Rockefeller Foundation in collaboration with local health officers, with the result that yellow fever by 1927 appeared to be all but eradicated from the Western Hemisphere.

Jungle Yellow Fever. In the years 1928–30, however, it was found that yellow fever was not abiding by the rules laid down from earlier experience. The cleaning up of urban foci no longer sufficed to control the disease. It became necessary in northeast Brazil to institute anti-aegypti measures in small towns and even rural districts. These measures proved effective and succeeded in eliminating the disease in that region in 1934. During these years another complexity in other localities was discovered in the form of "jungle yellow fever," a type of the disease found in regions of forests and uncleared land, and, what was more startling, in the complete absence of *Aedes aegypti*. Virus strains from these cases are identical immunologically and otherwise with the classical strains; the difference is epidemiological. Yellow fever in South America[128] as well as in parts of Africa is now regarded primarily as jungle infection with which *Aedes aegypti* is not associated, and in which infection of man is regarded as only incidental and not a part of the natural cycle of infection.

It was postulated that jungle animals serve as reservoirs for the virus, and extensive researches have been conducted to uncover the reservoir–vector cycle in both South America and Africa. Primates and marsupials have been suspected of serving as reservoirs, antibodies having been demonstrated in the blood of various monkeys and other animals. Virus has been recovered from wild-caught marmosets, and naturally infected jungle mosquitoes have been found.[129] In South America Haemogogus mosquitoes, and in Africa Aedes species, are incriminated. Many factors in maintenance and transmission of the jungle infection, including the transmitting vector for man, are not completely known. At the present time cases of jungle yellow fever in man represent potential sources of virus for the start of epidemics in urban areas where *Aedes aegypti* are present.

Field Survey. Two laboratory procedures have aided greatly in the survey work conducted by the Rockefeller Foundation in both South America and Africa. One is the serum neutralization test[130] using mouse-adapted virus. This test is specific and a neutralizing serum indicates previous infection with the

---

[128] Soper: Trans. Royal Soc. Trop. Med. Hyg., 1938–39, 32:297.
    [129] Smithburn and Haddow: Amer. Jour. Trop. Med., 1946, 26:261; Kumm, *et al.*: Amer. Jour. Hyg., 1946, 43:13.
    [130] Sawyer and Lloyd: Jour. Exper. Med., 1931, 54:533.

virus. Tests by this method have indicated the presence of yellow fever in regions in which it was thought to have disappeared, and by testing sera from persons of various ages in a community it has been possible to estimate at what date yellow fever was last present there.

The other important tool in field survey has been viscerotomy,[131] a procedure by which a portion of liver is taken post mortem from persons dying less than eleven days after the onset of any febrile illness regardless of the clinical diagnosis. Because of the characteristic lesion of yellow fever, histological examination of these specimens enables one to determine with considerable certainty whether the disease was yellow fever. Many thousands of liver specimens have been examined and the presence or absence of yellow fever determined in regions where it would be otherwise impossible.

The complement-fixation test,[132] in which infected tissue is the source of antigen, has been shown to be specific and is positive following clinical or subclinical illness. Unlike the neutralizing antibody, complement-fixing antibody does not persist indefinitely and is present in only a small percentage of vaccinated persons. It thus has value in detecting recent infection.

Active Immunization. Since the epidemiology of jungle yellow fever did not permit its control by the orthodox methods, resort to active immunization of man was necessary. For this purpose neutrotropic strains of virus which have lost their viscerotropism have been invaluable. At first mixtures of this virus and immune serum were given. At the present time a strain of virus, 17D, which has been maintained in tissue culture through many generations, is being used. Repeated transfer in tissue culture has resulted in a strain of virus whose neutropism and viscerotropism have both been depressed, and it has proved safe and suitable for human use.[133] Subcutaneous inoculation renders monkeys immune to potent strains, and in both monkeys and man neutralizing antibody appears in the serum. Field tests indicate that immunity in man appears not later than a week after inoculation and that vaccinated persons are protected while unvaccinated persons in the same groups may become infected. Antibodies indicative of resistance to infection are present in the serum of most immunized persons at least five years after vaccination.

The vaccine used is a frozen and dried emulsion of infected chick embryo which is rehydrated with a saline solution before use. It has been extensively used in South America and in Africa. Besides its use in natives of endemic regions, it is given to persons working with yellow fever or going into areas where it exists.

<center>DENGUE FEVER</center>

Dengue,[134] or *breakbone fever,* is an infectious disease found in tropical and subtropical climates. It is characterized by fever, headache, muscular pains and, in a large fraction of cases, a maculopapular rash. About 30,000 cases occurred in Galveston, Texas, during the summer and fall of 1922. An outbreak occurred in Florida in 1934. A high incidence among the affected population is common.

[131] Soper, Ricard and Crawford: Amer. Jour. Hyg., 1934, *19:*549.
[132] Perlowagora and Lennette: Amer. Jour. Trop. Med., 1944, *24:*235.
[133] Soper and Smith: Amer. Jour. Trop. Med., 1938, *18:*111.
[134] *Cf.* p. 349 of *Virus and Rickettsial Diseases.* (Ref. 64.)

The epidemiology of dengue presents a striking resemblance to that of yellow fever. It is transmitted by the yellow fever mosquito, *Aedes aegypti*, and by *A. albopictus* and *A. scutellaris*. Epidemics occur when there are simultaneously present cases of the disease, large numbers of *Aedes aegypti*, and many non-immune individuals. Prevention has been based on mosquito control.

The virus of dengue is present in the patient's blood during the last day of the incubation period and usually for the first three days of illness. Infective blood when inoculated either intravenously or subcutaneously into susceptible persons produces the disease.

Adaptation to the mouse, although difficult, has been successfully accomplished, and the passage virus proved to be of potential value as an immunizing agent.[135] Other laboratory animals appear to be insusceptible, although inapparent infections have been produced in monkeys.[136] Of epidemiologic interest is the fact that monkeys in endemic regions, like many persons, were immune, while monkeys brought in screened cages from other regions were susceptible. This may indicate an animal reservoir of the virus.

One attack of dengue confers an uncertain degree of immunity, second, third and even fourth attacks having been observed. However, this may be accounted for in part by immunologically different strains.

PHLEBOTOMUS FEVER[137]

This disease, also known as *pappataci fever, sandfly fever* and *three-day fever,* occurs during the hot dry season in many regions of the tropics and subtropics. It is a fever of short duration with clinical signs resembling influenza but without the signs of inflammation of the respiratory tract. One attack confers immunity for at least four months and probably for a year or more. The disease is transmitted, as far as is known, only by sandflies (pappataci flies, *Phlebotomus papatasii*) that have fed on the blood of patients, although there is a possibility of a type of congenital transmission in the fly. After feeding, the flies become infective on about the seventh day. Bedbugs and mosquitoes (*Culex pipiens* and *Aedes aegypti*) have been reported unable to transmit the infection.

No unquestionably susceptible experimental animal has been found and experiments have necessarily been done on human volunteers. A claim for cultivation of the virus in fertile eggs has not been confirmed in more recent work.

The virus is present in the blood of man in amounts up to 1000 infectious doses per milliliter one to two days before the onset of fever and twenty-four hours, but not forty-eight hours, thereafter. Serum remains infectious when stored in the cold or when frozen and dried. Differential filtration tests indicate that the virus has a probable diameter of 25 to 40 mμ.

Experimental transmission in man is accomplished much more readily by intravenous or intracutaneous inoculation than by subcutaneous or intramus-

[135] Sabin and Schlesinger: Science, 1945, *101;*640.

[136] Simmons, St. John and Reynolds: Philippine Jour. Sci., 1931, 44:1.

[137] For a general discussion of this disease and a preliminary report of investigations undertaken by an Army Commission see: Sabin, Philip and Paul: Jour. Amer. Med. Assn., 1944, *125;*603, 693.

cular. Subjects that have received subinfective doses by one of the latter routes have been shown to be immune to subsequent infection. Prophylaxis by control of the vector is difficult because the flies are able to pass through an ordinary mosquito net.

INFECTIOUS HEPATITIS[138]

This disease, which has no doubt been afflicting man for centuries, was until recently one of a poorly understood group of icteric conditions. Many cases have presumably been called "catarrhal jaundice" or "idiopathic acute yellow atrophy of the liver," depending in part upon the severity of the attack. It has also been confused with Weil's disease (p. 749), a leptospirosis. Mainly as the result of investigations stimulated by outbreaks during World War II, infectious hepatitis has now emerged as a well defined entity, clinically and etiologically.

The Disease. This is an acute febrile infectious disease characterized by symptoms referable in large part to liver damage, which is the outstanding pathological effect of the infection. Although clinical jaundice is typical, an unknown fraction of cases present the pre-icteric signs without developing jaundice. In the Mediterranean region during 1943 and 1944 infectious hepatitis occurred in high incidence in military personnel. In two and one-half years 35,000 cases were recognized in American troops, with epidemic peaks occurring in November and December. Transmission by contact is recognized but evidence has also been presented that water and food[139] may be the source of infection. These observations correlate with the demonstration of the infectious agent in feces of infected persons, and with the findings that poor sanitation is related to presence of the disease. The incubation period has been estimated at seven to thirty-eight days, and in experimental subjects was twenty to thirty days.

The Virus. Laboratory investigation has been seriously handicapped by the lack of a susceptible experimental animal. Nevertheless, by using human volunteers, several investigators have established that (1) the agent is filterable, (2) it has a relatively high heat tolerance, withstanding 56° C. for thirty minutes, (3) it is present in the serum and in the feces of patients in the acute stage, and (4) it is infective for man either by parenteral injection or by ingestion.

Evidence has been presented[140] that passive immunization during the incubation period with normal human gamma globulin will prevent the disease. The extended incubation period makes this disease particularly amenable to this type of treatment.

Serum Jaundice. One of the episodes that stimulated interest in hepatitis was the occurrence of jaundice in a large number of military personnel who had received yellow fever vaccine.[141] During 1941 and 1942 several million doses of yellow fever vaccine were supplied to the armed forces of the United

[138] *Cf.* review by Paul: Bull. New York Acad. Med., 1946, 22:204; Gauld: Amer. Jour. Hyg., 1946, 43:248 et seq.

[139] Read, *et al.*: Amer. Jour. Pub. Health, 1946, 36:367.

[140] Stokes and Neefe: Jour. Amer. Med. Assn., 1945, *127*:144.

[141] Sawyer, *et al.*: Amer. Jour. Hyg., 1944, 39:337; *40*:35 *et seq.*

The epidemiology of dengue presents a striking resemblance to that of yellow fever. It is transmitted by the yellow fever mosquito, *Aedes aegypti*, and by *A. albopictus* and *A. scutellaris*. Epidemics occur when there are simultaneously present cases of the disease, large numbers of *Aedes aegypti*, and many non-immune individuals. Prevention has been based on mosquito control.

The virus of dengue is present in the patient's blood during the last day of the incubation period and usually for the first three days of illness. Infective blood when inoculated either intravenously or subcutaneously into susceptible persons produces the disease.

Adaptation to the mouse, although difficult, has been successfully accomplished, and the passage virus proved to be of potential value as an immunizing agent.[135] Other laboratory animals appear to be insusceptible, although inapparent infections have been produced in monkeys.[136] Of epidemiologic interest is the fact that monkeys in endemic regions, like many persons, were immune, while monkeys brought in screened cages from other regions were susceptible. This may indicate an animal reservoir of the virus.

One attack of dengue confers an uncertain degree of immunity, second, third and even fourth attacks having been observed. However, this may be accounted for in part by immunologically different strains.

PHLEBOTOMUS FEVER[137]

This disease, also known as *pappataci fever, sandfly fever* and *three-day fever*, occurs during the hot dry season in many regions of the tropics and subtropics. It is a fever of short duration with clinical signs resembling influenza but without the signs of inflammation of the respiratory tract. One attack confers immunity for at least four months and probably for a year or more. The disease is transmitted, as far as is known, only by sandflies (pappataci flies, *Phlebotomus papatasii*) that have fed on the blood of patients, although there is a possibility of a type of congenital transmission in the fly. After feeding, the flies become infective on about the seventh day. Bedbugs and mosquitoes (*Culex pipiens* and *Aedes aegypti*) have been reported unable to transmit the infection.

No unquestionably susceptible experimental animal has been found and experiments have necessarily been done on human volunteers. A claim for cultivation of the virus in fertile eggs has not been confirmed in more recent work.

The virus is present in the blood of man in amounts up to 1000 infectious doses per milliliter one to two days before the onset of fever and twenty-four hours, but not forty-eight hours, thereafter. Serum remains infectious when stored in the cold or when frozen and dried. Differential filtration tests indicate that the virus has a probable diameter of 25 to 40 mμ.

Experimental transmission in man is accomplished much more readily by intravenous or intracutaneous inoculation than by subcutaneous or intramus-

---

[135] Sabin and Schlesinger: Science, 1945, *101*:640.

[136] Simmons, St. John and Reynolds: Philippine Jour. Sci., 1931, *44*:1.

[137] For a general discussion of this disease and a preliminary report of investigations undertaken by an Army Commission see: Sabin, Philip and Paul: Jour. Amer. Med. Assn., 1944, *125*:603, 693.

cular. Subjects that have received subinfective doses by one of the latter routes have been shown to be immune to subsequent infection. Prophylaxis by control of the vector is difficult because the flies are able to pass through an ordinary mosquito net.

<div align="center">

INFECTIOUS HEPATITIS[138]

</div>

This disease, which has no doubt been afflicting man for centuries, was until recently one of a poorly understood group of icteric conditions. Many cases have presumably been called "catarrhal jaundice" or "idiopathic acute yellow atrophy of the liver," depending in part upon the severity of the attack. It has also been confused with Weil's disease (p. 749), a leptospirosis. Mainly as the result of investigations stimulated by outbreaks during World War II, infectious hepatitis has now emerged as a well defined entity, clinically and etiologically.

The Disease. This is an acute febrile infectious disease characterized by symptoms referable in large part to liver damage, which is the outstanding pathological effect of the infection. Although clinical jaundice is typical, an unknown fraction of cases present the pre-icteric signs without developing jaundice. In the Mediterranean region during 1943 and 1944 infectious hepatitis occurred in high incidence in military personnel. In two and one-half years 35,000 cases were recognized in American troops, with epidemic peaks occurring in November and December. Transmission by contact is recognized but evidence has also been presented that water and food[139] may be the source of infection. These observations correlate with the demonstration of the infectious agent in feces of infected persons, and with the findings that poor sanitation is related to presence of the disease. The incubation period has been estimated at seven to thirty-eight days, and in experimental subjects was twenty to thirty days.

The Virus. Laboratory investigation has been seriously handicapped by the lack of a susceptible experimental animal. Nevertheless, by using human volunteers, several investigators have established that (1) the agent is filterable, (2) it has a relatively high heat tolerance, withstanding 56° C. for thirty minutes, (3) it is present in the serum and in the feces of patients in the acute stage, and (4) it is infective for man either by parenteral injection or by ingestion.

Evidence has been presented[140] that passive immunization during the incubation period with normal human gamma globulin will prevent the disease. The extended incubation period makes this disease particularly amenable to this type of treatment.

Serum Jaundice. One of the episodes that stimulated interest in hepatitis was the occurrence of jaundice in a large number of military personnel who had received yellow fever vaccine.[141] During 1941 and 1942 several million doses of yellow fever vaccine were supplied to the armed forces of the United

[138] *Cf.* review by Paul: Bull. New York Acad. Med., 1946, 22:204; Gauld: Amer. Jour. Hyg., 1946, 43:248 et seq.

[139] Read, *et al.*: Amer. Jour. Pub. Health, 1946, 36:367.

[140] Stokes and Neefe: Jour. Amer. Med. Assn., 1945, 127:144.

[141] Sawyer, *et al.*: Amer. Jour. Hyg., 1944, 39:337; 40:35 *et seq.*

States. Early in 1942 cases of jaundice began to be observed in increased in-
cidence, following vaccination by a period of weeks or months. The jaundice
was due to hepatitis and some cases were fatal. Cases had been observed pre-
viously in vaccinated individuals, and although the cause of the jaundice was
not fully determined, a change in the virus strain used in preparation of the
vaccine was apparently sufficient to remove this complication. Up to the end of
1942 there had been recorded 26,771 cases of "post-vaccination jaundice" in
the army, an estimated overall incidence of 18 per 1000 vaccinated persons.
Most of the cases, however, followed the use of only certain lots of vaccine, the
incidence of jaundice produced with some lots reaching 50 to 100 per 1000.
Investigation suggested that certain lots of human serum used in making the
vaccine were the responsible factor, and with the elimination of serum from
the vaccine cases of jaundice promptly stopped appearing.

Since this experience many instances have come to light of icteric disease fol-
lowing injection of human serum or plasma, or transfusions of blood. One of
the features which delayed recognition of the relation of serum injections to
this disease, now known as serum jaundice, is its lengthy incubation period.
Sixty to 150 days or more may elapse between the time of the inoculation and
the appearance of illness. In contrast to infectious hepatitis, in which the virus
has been demonstrated in the blood only during the acute stage, the agent of
serum jaundice may be present in the blood during the incubation period and
has been demonstrated there as long as two months before the appearance of
jaundice. Thus, such a carrier is of danger if he happens to be chosen as a
donor for blood, serum or plasma. Attempts have been made to render plasma
non-infective, for instance by ultraviolet irradiation,[142] but here again investiga-
tion is handicapped by the lack of a susceptible animal, necessitating the use
of human volunteers.

While the agents of infectious hepatitis and serum jaundice have much in
common, they are apparently not identical. In addition to the differences noted,
immunologic dissimilarity has also been observed. The exact relationship of
these two agents must await further research.

ENCEPHALITIS

Encephalitis, or inflammation of the brain, may be caused by a variety of
etiological agents. A number of human diseases of obscure nature are also in-
cluded under this general term, but due to lack of knowledge concerning
them, classification of these entities has not been satisfactory. The following
is an adaptation from a classification of the human encephalitides due to
viruses, and those suspected of having a virus etiology.[148]

I. Infectious encephalitis
    1. Type A. von Economo's, or lethargic encephalitis.
    2. Type B. (arthropod-borne).
        (a) St. Louis encephalitis
        (b) Equine encephalomyelitis (several types)
        (c) Japanese encephalitis
        (d) Russian encephalitis
    3. Other types.
II. Post-infectious, or para-infectious encephalitis.

[142] Oliphant and Hollaender: Pub. Health Repts., 1946, *61*:598.
[148] Report on the St. Louis Outbreak of Encephalitis, Pub. Health Bull. No. 214, 1935.

Lethargic Encephalitis.[144] von Economo described an epidemic of encephalitis which occurred in Vienna during the winter of 1916–17. This marked the beginning of recognized outbreaks of this disease, termed *lethargic encephalitis,* which appeared in many parts of the world. The early outbreaks seemed to be associated to some extent with epidemics of influenza but the etiology of the disease is not yet established. In the quest for a causative agent interest has centered on various bacteria, chiefly neurotropic strains of *Streptococcus viridans,* and to a greater extent upon viruses. In 1920 Levaditi and Harvier isolated from patients a strain of herpes virus which was capable of producing encephalitis in rabbits, and which they considered to be the cause of the human disease. One of the patients was suffering from herpes, however, and the possibility remains that this was the source of the virus. Other workers have obtained similar results, and herpes virus has been found in brain tissue, nasopharynx, spinal fluid and saliva of cases. The successful isolations are relatively few compared to unsuccessful attempts, and doubt is cast on the significance of this virus by the fact that herpes virus has been isolated also from spinal fluid and saliva of persons without signs of either encephalitis or herpes. Whatever the relation of herpes virus to von Economo encephalitis, it is nevertheless apparent that herpes, or herpes-like viruses, can on occasion produce central nervous system infection in man. The B virus from a case of human myelitis and the demonstration of herpes virus in certain other cases of human encephalitis (p. 863) are illustrations.

The Arthropod-Borne Encephalitides. In recent years there has emerged a group of human and animal diseases caused by neurotropic viruses which have in common a similar but not identical epidemiological pattern, and are transmitted by various kinds of arthropods, *viz.,* mosquitoes, ticks and mites. The total picture of this group is far from complete, but it seems apparent that at least several of these infections are primarily diseases of animals, occurring in inapparent form, with infection of man being only incidental to the natural cycle of transmission. Included in this group are equine encephalomyelitis, St. Louis encephalitis, Japanese encephalitis, Russian encephalitis, louping ill, and several less studied agents that have been encountered in various parts of the world.

Equine Encephalomyelitis.[145] During an epizootic of this disease in California in 1930 a filterable virus was recovered[146] from the brains of affected horses, which proved to be quite distinct from other known neurotropic viruses, including the previously described virus of Borna disease, a European encephalomyelitis of horses. Since then equine encephalomyelitis has been described in most regions of the United States and in parts of Canada, in South and Central America, and in Russia and Japan. During 1937 and 1938, when large epizootics occurred, more than a quarter million cases in equines were recorded, with a total mortality rate of over 20 per cent. A severe form of the disease occurs in the eastern part of this country and the "eastern type" virus recovered from these cases is immunologically distinguishable from the originally described less severe "western type." A "Venezuelan type" has also been

[144] *Epidemic Encephalitis,* Third Report of the Matheson Commission. Columbia University Press, New York. 1939.
[145] Kelser: Ann. New York Acad. Sci., 1947, 48:385.
[146] Meyer, Haring and Howitt: Science, 1931, 74:227.

isolated from several parts of South America, and is immunologically distinct from the other two.[147] Infection of man has occurred with all three strains.

Animal Susceptibility. Although these viruses were first observed in horses, thereby supplying the name universally used, equines are not the only animal host, and probably not the primary one, for these strains. The eastern type has been recovered from the brains of pigeons[148] found suffering from an epizootic at the time equine encephalomyelitis was prevalent in the same region, and several reports[149] have appeared of the demonstration of the virus in the brains of pheasants. A great variety of animals have been infected by artificial inoculation of the virus. In addition to the usual laboratory animals (mice, rats, guinea pigs, rabbits and monkeys) calves, goats, pigeons, guinea fowl, young puppies, and several species of wild rodents are susceptible to the western strain. Sheep, pigs and cats are apparently resistant to the western but susceptible to the eastern strains. Using the Argentine strain, immunologically identical with western virus, Remlinger and Bailly[150] found a number of birds susceptible, including the mallard duck and other migratory species, a finding of possible significance in the natural distribution of the virus. Quail, sparrows, chicks, hedgehogs and goats can be infected with the eastern strain. Infection of turkeys, chickens and other birds, manifest only by the presence of virus in the blood stream, has been described. In extensive tests on sera of animals and birds taken in endemic areas neutralizing antibody against the western strain was found in many cases, but this as well as other types of evidence point most strongly to the domestic chicken as the important reservoir of infection in the areas most intensively studied in the Far West.[151] There are grounds for suspecting that the reservoir host and arthropod vector may vary from one region to another.

The Virus. Equine encephalomyelitis virus has been demonstrated in the blood of horses and guinea pigs during the febrile period preceding the onset of nervous system signs, but not after defervesence and the appearance of paralysis, when it is found in nervous tissue. After subcutaneous or intravenous inoculation in guinea pigs it may be demonstrated in the blood stream and appears to invade the brain by migration through vessel walls.[152] Once in the brain it may follow nerve tracts. The pathogenesis in young mice (fifteen days) is similar, but in slightly older mice (twenty-one days) there is evidence that the virus uses peripheral nerves as a path to the central nervous system.

Early estimations of the size of this virus, by differential filtration and centrifugation, indicated it was between 20 and 40 mμ in diameter. More recent study[153] assigns a diameter of approximately 50 mμ to both the eastern and western strains and electron micrography reveals circular images of uniform size.

A liponucleoprotein complex with an estimated molecular weight of 152

[147] Kubes and Rios: Science, 1939, 90:20.
[148] Fothergill and Dingle: Science, 1938, 88:549.
[149] van Roekel and Clarke: Jour. Amer. Vet. Med. Assn., 1939, 94:466.
[150] Remlinger and Bailly: Compt. Rend. Soc. Biol., 1936, 121:146.
[151] Cf. review by Hammon and Reeves: Amer. Jour. Pub. Health, 1945, 35:994.
[152] King: Jour. Exp. Med., 1939, 69:675.
[153] Sharp, et al.: Arch. Pathol., 1943, 36:167.

million has been isolated from infected tissues and shown to behave as the virus.[154]

Infectivity for Man. In the late summer of 1938 while equine encephalo-myelitis was prevalent in Massachusetts and Rhode Island, an epidemic of encephalitis comprising approximately forty cases, mainly in children, appeared in the same region. From the brain tissue of several cases coming to autopsy a filterable virus was recovered[155] by mouse inoculation which, from clinical, histological and immunological evidence in experimental animals, proved to be the eastern type of the horse virus. Human infection with equine encephalomyelitis had, in fact, been suspected as early as 1932 by Meyer, who observed an unusual type of encephalitis in three men who were in close contact with horses infected with the western type of the disease. Human infection with the western type was definitely proved by isolation[156] of the virus from man during an epidemic in California in 1938. Since then epidemics have been recognized in various parts of the West, often in conjunction with cases of St. Louis encephalitis.

Surveys by means of the serum neutralization test in the region of the Massachusetts epidemic and in various localities where the western type has occurred have furnished further information concerning human infection with this virus. These observations indicate that the presence of antibody in man usually results from an attack of the disease, although in some regions neutralizing sera have been obtained from persons with no history of nervous system disease. These findings are in contrast to the appreciable percentages of sera found in the general population capable of neutralizing St. Louis and certain other neurotropic viruses. All of 14 sera from patients with encephalitis in Massachusetts (eastern type) showed neutralizing ability, but 114 sera from contacts and other persons were negative. The early appearance of neutralizing antibodies (six to eight days) in human infection with the equine virus increases the potential value of the serum neutralization test as a diagnostic procedure.

Transmission.[157] The occurrence of outbreaks of this disease in late summer, as well as other epidemiological observations, suggested insect transmission, and the presence of virus in the blood of horses and experimental animals is compatible with this view. Kelser (1933) demonstrated the experimental transmission of the western strain to guinea pigs and to a horse by the bites of *Aedes aegypti* mosquitoes which had been allowed to feed upon infected guinea pigs. This was confirmed by Merrill and Ten Broeck[158] who also reported that the virus multiplies within the mosquito. A number of other species of Aedes, as well as the wood tick *Dermacentor andersoni,* have been found capable of transmitting the disease. During an outbreak in the Yakima Valley, Washington, in 1941, and in subsequent years Hammon[159] and his colleagues have repeatedly recovered this virus from the mosquito *Culex tarsalis,* caught

[154] Taylor, et al.: Jour. Inf. Dis., 1943, 72:31.
[155] Fothergill, Dingle, Farber and Connerley: New England Jour. Med., 1938, 219:411.
[156] Howitt: Amer. Jour. Pub. Health, 1939, 29:1083.
[157] *Cf.* Hammon: Amer. Jour. Trop. Med., 1948, 28:515.
[158] Merrill and Ten Broeck: Jour. Exp. Med., 1935, 62:687.
[159] Hammon, et al.: Jour. Inf. Dis., 1942, 70:263 *et seq.*

in the locality of the outbreaks, and from several other species less often. Transmission in the laboratory has been demonstrated with several species of the genera Culex and Culiseta.[160] C. *tarsalis* is especially prone to feed on chickens, which is added evidence for its role in transmission.

Mosquitoes have also been incriminated on epidemiological grounds as vectors of Venezuelan encephalomyelitis, and several potential vectors have been demonstrated.

Western virus has also been recovered from chicken mites (*Dermanyssus gallinae*), giving evidence that this species transmits the virus within the avian reservoir.[161] Recovery of virus from wild bird mites (*Liponyssus bursa*) is also reported. Assassin bugs (*Triatoma sanguisuga*), collected in an epizootic region, have been found naturally infected, and experimental transmission has been accomplished with these insects. This species also may be one of the arthropod vectors effecting transmission within the animal reservoir.[162]

Artificial Immunization. Early attempts to induce active immunity showed that guinea pigs could be protected to some degree by a variety of preparations, the efficacy of which depended mainly upon the amount of residual active virus present in the preparation. Shahan and Giltner, and Cox and Olitsky[163] reported successful immunization with formolized preparations in which no active virus could be detected by various tests. An advance was made when this method of inactivation was applied to the virus as cultured in chick embryos. It had previously been found that chick embryos are quite susceptible to this virus, the titer of infected embryo tissue being considerably higher than that of horse or guinea pig brain. It has been estimated that 3,000,000 horses and mules, or about one-fifth of the total number in the United States, received prophylactic inoculations during 1939. Some of these were no doubt inoculated during the incubation period of the disease. Nevertheless the incidence was reported as 1.2 per 1000 in unvaccinated and 0.37 in vaccinated horses and mules.

This type of vaccine has also been employed in immunizing laboratory workers, since a number of cases of infection and death have occurred in persons working with this virus in laboratories.

Passive immunization has also been demonstrated. If an immune rabbit serum of high titer is used mice and guinea pigs can be protected from death even when the serum is withheld until a considerable interval after the virus is injected.[164]

St. Louis Encephalitis. Cases of a disease now known as St. Louis encephalitis first appeared in Paris, Illinois, in the summer of 1932, and were diagnosed as von Economo encephalitis. A larger outbreak occurred in the late summer of the following year in the vicinity of St. Louis, Missouri, and these cases were recognized as a new clinical entity. The 1932 cases were shown in retrospect to be this new disease. The disease appeared again in St. Louis in 1937, and smaller outbreaks have since been observed in other western states, often associated with western equine encephalomyelitis. There is indi-

[160] Hammon and Reeves. Jour. Exp. Med., 1943, 78:425.
[161] Sulkin: Science, 1945, 101:381.
[162] Grundmann, et al.: Jour. Inf. Dis., 1943, 72:163.
[163] Cox and Olitsky: Jour. Exp. Med., 1936, 63:754.
[164] Zichis and Shaughnessy: Jour. Amer. Med. Assn., 1940, 115:1071.

rect evidence from the serum neutralization test that the infection is present in many parts of the United States. An early survey[165] indicated that 36 per cent of contacts and 9 per cent of non-contacts possess neutralizing serum. In an endemic locality (Yakima Valley, Washington) the incidence of positive sera in persons who have resided there some time is near 80 per cent.[166] Outbreaks of this disease have occurred in the summer but disappear with the advent of autumn. In the 1933 outbreak in St. Louis cases appeared first outside the city, and the morbidity rate for the epidemic was actually higher in the rural districts surrounding the city than it was in the urban area into which the disease later moved. There is no evidence that St. Louis encephalitis is spread by milk, water or food supply, and attention was early turned to the possibility of insect vectors. Experiments with mosquitoes by the first investigators were negative; since then conclusive evidence has been presented that mosquitoes and other arthropods act as vectors (*vide infra*).

The Virus. Muckenfuss, Armstrong and McCordock in 1933 successfully infected monkeys by intracerebral inoculation of brain tissue of a patient who had succumbed to the disease. Shortly afterward Webster and Fite found that mice are susceptible to intracerebral inoculation, and mice have remained the animals of choice for the investigation of this disease. The infectious agent was shown to be filterable and its relation to the human disease was confirmed by serum neutralization tests with the serum of recovered patients, 95 per cent showing protection. Multiplication of the virus occurs in tissue culture and upon the chorioallantois of fertile eggs.[167]

This virus is somewhat labile at room temperature but deterioration may be minimized by using normal serum in the diluent. It shows partial immunologic relationship to West Nile virus (p. 903) and Japanese encephalitis virus, especially by the complement-fixation test.

Reservoir of Infection. Beside mice, hamsters are susceptible and a number of other animals and birds have been shown to suffer an inapparent infection after inoculation, detectable by the presence of circulating virus in the blood. Among these is the horse, which fact provides another similarity between this virus and those of equine encephalomyelitis. Evidence from neutralization tests on serum taken in endemic areas points to the chicken as the reservoir of infection,[151] as was described above for western equine encephalomyelitis. As with the latter virus chickens develop an inapparent infection with viremia. In fact, all the information at hand regarding the St. Louis and western equine viruses indicates a remarkable parallelism between the two agents. The St. Louis virus has also been recovered from captured *Culex tarsalis* and other species, and laboratory transmission is reported with species of Culex and Aedes. All observed epidemics are said to have occurred in the presence of large numbers of mosquitoes, those in the West being associated particularly with irrigated regions.

Since the virus does not survive over the winter in the mosquito, and is present for short periods only in the chicken, it was natural that search be made for another reservoir capable of maintaining the virus over long periods. Such a

[165] Wooley and Armstrong: Pub. Health Repts., 1935, 49:1495.
[166] Hammon: Jour. Amer. Med. Assn., 1943, 121:560.
[167] Harrison and Moore: Amer. Jour. Path., 1937, 13:361.

reservoir appears to have been discovered in the chicken mite (*Dermanyssus gallinae*) which has been found naturally infected, and in which congenital transmission from one generation in the laboratory to the next has been accomplished, as well as transmission of infection to chickens by the bites of mites.[168] The tick, *Dermacentor variabilis*, is also capable of transmission in the laboratory,[169] and congenital passage in the tick occurs.

Artificial Immunization. Mice may be immunized by moderate doses of active virus given intraperitoneally and immune serum has been produced in non-susceptible animals such as the rabbit. A formalin-inactivated vaccine, capable of protecting mice against peripheral inoculation of virus (and against intracerebral inoculation if large and repeated immunizing doses are given) has been described.[170] Although the vaccine was shown practicable for use in man, and capable of inducing neutralizing antibody in about one half of the inoculated subjects, no field trials have been reported. A vaccine rendered non-infective by exposure for short intervals to intense ultraviolet radiation has also been described.[171] In the dosages used it produced a high degree of immunity in mice.

Japanese Encephalitis. A type of encephalitis, occurring in the summer, has been recognized as a clinical entity in Japan for many years, first appearing in epidemic form during the summer and fall of 1871. Cases have also been reported from China, Formosa, Korea, Manchuria, Okinawa and in the far eastern districts of the U.S.S.R. Since first recognized, outbreaks of this disease have occurred repeatedly in Japan. The epidemiological features[172] are similar to those of the St. Louis type of encephalitis, and differ from those of von Economo's encephalitis. The summer incidence raised the suspicion of insect transmission in this disease also, and four species of culicine mosquitoes were reported by Japanese workers[173] to become infected when allowed to bite patients. Isolation of the virus from wild culicine mosquitoes has been reported. More recently seven species studied in Hammon's laboratory[174] were found capable of experimental transmission, and the possibility of Japanese encephalitis becoming established in this country seems clear.

The virus was first transmitted to monkeys by Hayashi who used brain tissue from a fatal case in the 1934–35 epidemic. Later other workers established infection in mice with other strains of the virus.[175] The various strains of Japanese encephalitis have been shown to be identical, but in spite of the epidemiological and clinical similarity between this disease and St. Louis encephalitis, the two viruses differ antigenically.[176] Nevertheless there is some minor antigenic relationship, these two agents together with West Nile virus (p. 903) forming a subgroup of neurotropic viruses. A difference between Japanese and St. Louis virus is seen also in the range of susceptible animals. A

---

[168] Smith, Blattner and Heys: Jour. Exper. Med., 1948, 87:119.

[169] Blattner and Heys: Jour. Exp. Med., 1944, 79:439.

[170] Sabin, et al.: Jour. Amer. Med. Assn., 1943, 122:477.

[171] Levinson, et al.: Jour. Amer. Med. Assn., 1944, 125:531.

[172] Cf. Warren: Amer. Jour. Trop. Med., 1946, 26:417.

[173] Mitamura, et al.: Trans. Soc. Path. Jap., 1937, 27:573; ibid., 1938, 28:135.

[174] Reeves and Hammon: Jour. Exp. Med., 1946, 83:185.

[175] Kawamura, et al.: Kitasato Arch. Exp. Med., 1936, 13:281.

[176] Webster: Jour. Exp. Med., 1938, 67:609.

severe infection occurs in monkeys with the Japanese type, while the reaction in these animals to St. Louis virus is mild or absent. In sheep the latter virus is innocuous but Japanese virus produces a severe infection.

Formalin-inactivated vaccines made from both infected mouse brain and from infected chick embryos have been used.[177] Sera can be examined by the neutralization or complement-fixation test. Such tests, applied to animal

Fig. 253. Pathologic changes resulting from Japanese encephalitis infection. Above: Substantia nigra from a human case; foci of mononuclear cell infiltration are present, and numerous ganglion cells are in a disintegrated state or have disappeared. Hematoxylin and eosin; × 90. Below: Region of superior temporal gyrus from a human case; a vein at the cortico-medullary junction is surrounded by a "cuff" of lymphocytes. (Haymaker and Sabin: Arch. Neurol. Psychiat., 1947; courtesy Army Institute of Pathology, accession no. 156880.)

serums from Okinawa following an epidemic, revealed antibody in the blood of horses.[178] There is reason to believe that animals may serve as a reservoir for the virus with arthropod transmission to man, in the same manner as has become evident for the summer encephalitides in the United States. Although Japanese encephalitis has not been recognized in this country the sera of many Americans neutralize the virus.

[177] *Cf.* Warren: Jour. Immunol., 1947, 58:211.
[178] Hodes, Thomas and Peck: Science, 1946, 103:357.

Russian Tick-Borne Encephalitis.[179] (Synonyms: Vernal, Spring-Summer, Forest-Spring, Far Eastern, encephalitis.) This form of encephalitis was recognized in 1937 among residents of the far eastern provinces of the Soviet Union. It has a seasonal incidence, occurring from May to August, is found in wooded districts and attacks especially persons who do forestry work. It is transmitted by the bite of infected ticks, especially *Ixodes persulcatus,* and congenital transmission of the virus occurs in this tick. On this score as well as by antigenic relationship it resembles louping ill (below), a tick-transmitted virus disease of sheep. Mice, sheep, monkeys and guinea pigs are susceptible, in varying degrees. Numerous forest rodents and domestic animals in endemic regions have yielded neutralizing sera, some have been found naturally infected and others have been proved susceptible by inoculation. There thus appears to be a widespread animal reservoir in addition to the tick. Many persons in endemic regions have antibody in their serum without a history of the disease, indicating that unrecognized infections occur in man.

Successful protection of man in an endemic area has been reported[180] by the use of a formalin-inactivated vaccine.

Other Types of Infectious Encephalitis. *Australian X Disease.* In 1917 and 1918 a disease resembling Japanese encephalitis appeared in epidemic form in Australia. An agent which appeared to be a filterable virus was isolated and found to be infectious for monkeys, sheep, cattle and horses. Unfortunately it was not possible to maintain the virus indefinitely, and thorough investigation has not been possible. Similar epidemics occurred in 1922 and 1926 but further strains were not isolated. The similarity of this agent to that of louping ill was noted, but it now seems probable that the disease actually was Japanese encephalitis.

Louping Ill.[181] This is a disease primarily of sheep, occurring in Scotland and the northern counties of England, and characterized by encephalomyelitis. It has also been reported in the U.S.S.R. The virus is infectious also for monkeys and mice, resembling in this respect the agent of Japanese encephalitis. Perdrau[182] has compared histological material from louping ill and the Australian disease and found the lesions very similar, the characteristic change in each case being necrosis of the Purkinje cells of the cerebellum. This lesion is apparently responsible for the ataxia seen in infected monkeys and sheep. Another similarity to the Australian virus is seen in the infectivity of louping ill for cattle and horses.

Louping ill is transmitted in nature by a tick, *Ixodes ricinus,* and the virus is present in the blood stream during the febrile stage. Animals are susceptible to intranasal inoculation, and several infections in laboratory workers engaged in study of louping ill have been attributed to this virus. These cases were of varying degrees of severity, and although louping ill virus was not isolated from the cases, the diagnosis was made by means of the neutralization test, using serum taken after recovery.

---

[179] Cf. monograph by Silber and Soloviev: Amer. Rev. Soviet Med., 1946 (Special Supplement).
[180] Smorodintseff, et al.: Arch. ges. Virus forsch., 1941, 2:1.
[181] Cf. p. 701 of *Virus and Rickettsial Diseases.* (Ref. 64.)
[182] Perdrau: Jour. Path. Bact., 1936, 42:59.

This virus has a definite antigenic relationship to the virus of Russian en
cephalitis and it is noteworthy that both are transmitted by ticks.

West Nile Virus. In 1940 the isolation of a virus was reported[183] from the
blood of a native of Uganda suffering from a febrile illness. During con-
valescence the patient developed antibody against the isolated agent. Serum
tests on natives indicate the presence of this virus in several regions of Africa.
The virus is neurotropic, infective for mice and rhesus monkeys and has an es-
timated particle diameter of 21 to 31 mμ. It is antigenically related to the
viruses of St. Louis and Japanese encephalitis.

Several other filterable agents isolated from mosquitoes have been described.
Among these are the Semliki Forest virus[184] found in Uganda, and the Ilheus
encephalitis virus from Brazil.[185]

Relationships of the Viral Encephalitides. Investigation in this group
of viruses has been intense in recent years and certain relationships and gen-
eralities are becoming apparent. It was indicated above that these agents are
transmitted by arthropods, and appear primarily to affect animals with infec-
tion of man being only incidental.

Antigenic relationships are apparent among the St. Louis, Japanese and
West Nile viruses when tested by neutralization[186] or complement-fixation
tests. The equine encephalomyelitis strains stand apart antigenically, as do
Russian encephalitis and louping ill, the two latter being closely related anti-
genically.

Laboratory Diagnosis. This consists either of (1) isolation of the agent in a
suitable experimental animal from blood, spinal fluid or nervous tissue, or (2)
demonstration of an increase in serum antibody during convalescence. A serum
specimen should be taken as early as possible during the illness to compare
with one or more specimens taken during convalescence. Antibodies detectable
by the neutralization test are apparently present for long periods after infection.
The complement-fixation test[187] remains positive for shorter periods of time.

Postinfectious Encephalitis. This is a central nervous system disturb-
ance seen occasionally after various infections such as measles, varicella, in-
fluenza and vaccinia. Antirabies prophylaxis is sometimes followed by a similar
condition. Although this clinical entity has been called encephalitis, the char-
acteristic lesion is not an inflammation. The predominant pathologic change in
the nervous tissue is perivascular demyelinization, a lesion quite different from
those due to the known neurotropic viruses.

The etiology of these cases is obscure, but since they seem to follow virus in-
fections, it has been suggested that they are due to virus strains which have
suddenly gained the ability to invade the central nervous system. Against this
theory are the facts that efforts to recover virus from the brain of fatal cases
have failed, that the lesions, as mentioned above, do not resemble those due to
known viruses, and that similar encephalitides are seen after infections due to
agents other than viruses. A second hypothesis is that a latent virus has been

[183] Smithburn, *et al.*: Amer. Jour. Trop. Med., 1940, 20:471.
[184] Smithburn and Haddow: Jour. Immunol., 1944, 49:141.
[185] Laemmert and Hughes: Jour. Immunol., 1947, 55:61.
[186] Smithburn: Jour. Immunol., 1942, 44:25.
[187] Casals and Palacios: Jour. Exp. Med., 1941, 74:409.

stirred to activity by the infection, but this seems similarly untenable. Severe lesions of this character can be produced in animals after repeated peripheral injection of nervous tissue, or by injection of such tissue mixed with certain adjuvants.[188]

POLIOMYELITIS (EPIDEMIC INFANTILE PARALYSIS)

This disease[189] was described by Heine in 1840, and several epidemics in the latter part of the nineteenth century were studied intensively. It has since become prevalent in many parts of the world. Primarily a disease of children, data collected in recent years indicate an increased number of cases in older age groups. The prodromal stage may take the form of a general illness with
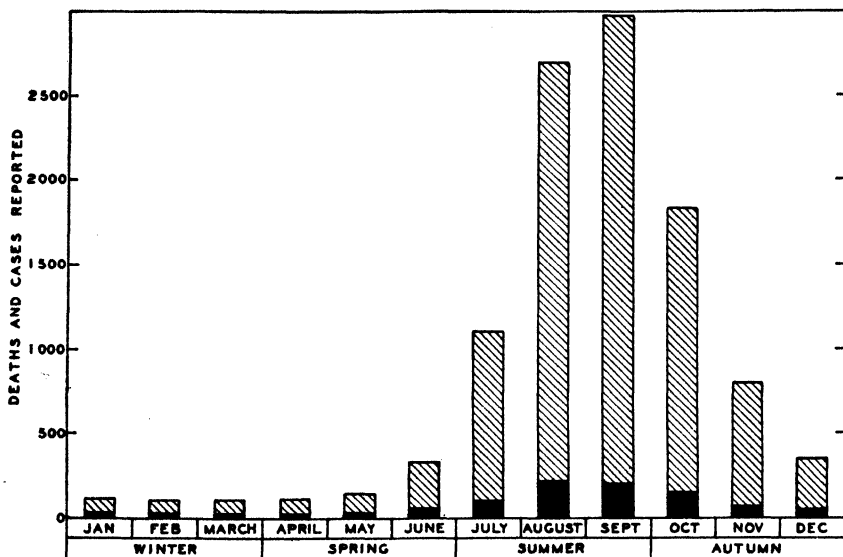
Fig. 254. The seasonal incidence of poliomyelitis. Averages of reported cases by months for the years 1939 to 1945 inclusive. Data from Supplements to Public Health Reports.

headache and fever. Meningeal signs appear and other evidence of beginning central nervous system involvement may be present. Many cases progress no farther than this (non-paralytic poliomyelitis) or may show transient weakness of a member. The classical case goes on to varying degrees of paralysis from which recovery is slow and sometimes is never complete. The most striking lesion in a paralytic case is destruction of the large nerve cells in the anterior horn of the spinal cord (Fig. 256). These cells give rise to the motor fibers of the peripheral nerves and their destruction results in flaccid paralysis. Inflammation in the region attacked is evidenced by congestion, cellular infiltration of the gray matter of the cord, perivascular cellular infiltration (illustrated for another neurotropic virus infection in Fig. 253), and phagocytosis of the necrotic motor cells (neuronophagia).

[188] Freund, Stern and Pisani: Jour. Immunol., 1947, 57:179.
[189] See the following reviews: *Poliomyelitis.* Williams and Wilkins Company, Baltimore. 1932; *Infantile Paralysis: a Symposium.* The National Foundation for Infantile Paralysis, New York City. 1941; Rhodes: Bull. Hyg. 1947, 22:353.

The Virus. Landsteiner and Popper were able to transmit the disease to monkeys in 1908 by intraperitoneal inoculation of spinal cord from a fatal case. Rhesus monkeys (*Macaca mulatta*) have been used to the greatest extent and are uniformly susceptible to established strains injected intracerebrally, but infection by other routes is somewhat irregular. Several other species may be infected also, including the chimpanzee which is infected by feeding upon virus-containing food. Marked differences in virulence between strains have been noted, and antigenic differences are also reported. It was early shown that the causative agent would pass bacterial filters and subsequent observations have indicated that it has the usual properties of a filterable virus.

Susceptibility of Rodents. In spite of repeated attempts to infect other laboratory animals, monkeys remained for some time the only susceptible host besides man. In 1939 Armstrong[190] reported that a strain of poliomyelitis virus (Lansing strain), which had been established in monkeys, would produce flaccid paralysis in cotton rats (*Sigmodon hispidus hispidus*) and could be transmitted from rat to rat by inoculation of brain tissue. Passage to laboratory mice was then successfully attempted and the mouse-established strain has proved of considerable value in investigations of this virus. Many other attempts have been made to adapt monkey or human virus to rodents but only a limited number have been successful.

Physical Properties. The virus of poliomyelitis has been described as one of the smallest, its size lying between 8 and 12 mμ when determined by differential filtration. This method assumes, however, that the particle under measurement is essentially spherical in shape. There is a possibility that the virus of poliomyelitis is cylindrical or filamentous in form with the smaller dimension being 12 to 15 mμ and the other much greater. Gard[191] has described the isolation of such a component from human poliomyelitic stools and from the brains of mice infected with the closely related Theiler's virus (p. 910). Both he and Melnick[192] have observed such filaments in concentrated preparations of virus when viewed with the electron microscope. The fact that a similar component can be isolated from normal stools (although not from normal brain tissue), and that a claim for a spherical shape of the virus has appeared,[193] indicates the uncertainty in interpretation of these findings.

The virus activity of tissue emulsions can be precipitated out with $(NH_4)_2SO_4$ and concentrated by appropriate procedures. The evidence suggests that the virus activity resides in a protein fraction.[194] Compared with certain others, the virus of poliomyelitis is relatively stable. It remains active over a wide range of hydrogen ion concentrations and in the presence of ether. It is resistant to x-rays and sonic vibrations, but relatively susceptible to heat, ultraviolet light and oxidizing agents. It may be stored for long periods of time in 50 per cent glycerol in the refrigerator.

Observations upon infected monkeys indicate that poliomyelitis virus travels by way of nerve tracts. This is true in the case of inoculation of peripheral

[190] Armstrong: Pub. Health Repts., 1939, 54:1719.
[191] Gard: Acta Med. Scandinavica, 1943, Supp. 143.
[192] Melnick: Jour. Immunol. Virus Res. & Exp. Chemotherap., 1944, 48:25.
[193] Loring, Marton and Schwerdt: Proc. Soc. Exp. Biol. Med., 1946, 62:291.
[194] Racker: Science, 1942, 96:364.

nerves as well as within the central nervous system in which migration of
virus has been traced both by animal test of various tissues and by histological
observations.

Epidemiology.[195] Poliomyelitis occurs both sporadically and epidemically
and is most common in the late summer and early autumn (Fig. 254). Peaks
of high incidence in the U. S. have occurred in 1916, 1931, 1944 and 1946.
In the latter year some 25,000 cases were reported. In California, the state
reporting the greatest number of cases in 1943, the case incidence reached 38.8
per 100,000 inhabitants, but it stays usually below 15 per 100,000 in the coun-
try as a whole. The reported case fatality, although varying in different out-
breaks, has in general declined since epidemics were first observed. This is
due at least in part to increased recognition of mild nonparalytic cases which,
of course, influences also the figures for morbidity and percentage of paralytic
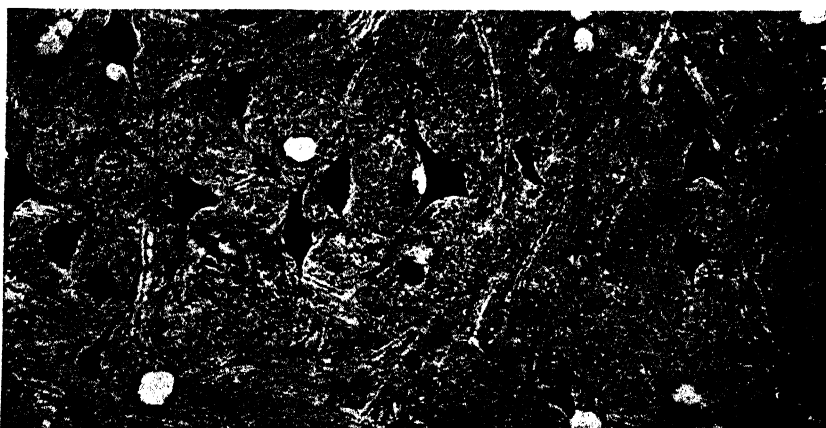
Fig. 255. Section through the anterior horn of the spinal cord of a normal monkey. This
is for comparison with Fig. 256. Hematoxylin and eosin; × 125.

cases. The case fatality figures for certain epidemic years in Massachusetts[196]
are: 1916, 23.5 per cent; 1927, 14.2 per cent; 1935, 4.4 per cent.

Transmission. The early epidemiological observations suggested that trans-
mission takes place by contact with cases and carriers, and it was supposed that
the upper respiratory tract is the portal of entry and exit of the virus. The find-
ing of virus in nasopharyngeal washings of cases and contacts[197] supported this
theory, and further studies have accumulated evidence that virus is present
in the throat or mouth, and perhaps in the nose, of cases and contacts. Virus
has been demonstrated in the tissues of the pharynx and ileum in fatal human
cases but rarely from other tissues except those of the nervous system.[198] Ex-
periments with cynomolgus monkeys also indicated that virus was present in
the pharyngeal and intestinal wall as well as the central nervous system.
The presence of virus in the throat in unrecognized infections[199] probably

[195] *Cf.* p. 555 of *Virus and Rickettsial Diseases.* (Ref. 64.)
[196] Legg: New Eng. Jour. Med., 1937, 217:507.
[197] Flexner, Clark and Fraser: Jour. Amer. Med. Assn., 1913, 60:201.
[198] Sabin and Ward: Jour. Exp. Med., 1941, 73:771; 74:519.
[199] Howe and Bodian: Amer. Jour. Hyg., 1947, 45:219.

explains the significant correlation between cases of bulbar poliomyelitis and tonsillectomy performed during epidemics. Apparently virus enters through the wound and goes directly to the brain stem by way of the nerves supplying the traumatized region.

Early investigators[200] reported finding virus in the feces of cases, but not until some years later was it generally realized that large amounts of virus (in monkey-infectious doses) are excreted in the feces of recognized cases and also in large numbers of persons with essentially subclinical disease. Such persons are found in epidemic areas and the greatest numbers are encountered among those who have had contact with a recognized case.[201] Virus has been detected repeatedly in sewage from areas where the disease is present.[202]

Fig. 256. Section through the anterior horn of the spinal cord of a monkey paralyzed with poliomyelitis. Destruction of large anterior horn cells has occurred and neuronophagia of the debris is apparent. Compare with the normal spinal cord of Fig. 255. Hematoxylin and eosin; × 125.

Whether transmission is usually effected with virus from the feces or from the throat is not now known; presumably both sources serve on occasion. The presence of virus in sewage represents a potential source of contamination of water supplies, and the existing reports on the subject suggest that poliomyelitis virus is less readily removed from contaminated waters than are bacterial pathogens. Actually, however, epidemics of poliomyelitis do not have the characteristics of water-borne epidemics and it seems unlikely that contaminated water accounts for very many cases. In recent years epidemiologic observations combined with laboratory tests have indicated that contact transmission plays an important role during epidemics.

The seasonal incidence of cases has suggested that insects may play a role in transmission. Although the stable fly, *Stomoxys calcitrans,* was at one time incriminated on the basis of successful transmission experiments, repetitions of the experiments produced negative results. Attempts to infect lice, fleas and

200 Kling, Wernstedt and Pettersson: Ztschr. f. Immunitätsf. u. Exper. Therap., 1911–12, 12:316, 657.
201 Gordon, et al.: Jour. Inf. Dis., 1948, 82:294.
202 Melnick: Amer. Jour. Hyg., 1947, 45:240.

several species of mosquitoes with laboratory strains have been unsuccessful although the virus was once recovered from bedbugs seven days after feeding. Several successful attempts to demonstrate virus in flies caught in epidemic areas have been reported.[203] These were largely blowflies, houseflies and similar types. Whether flies play a significant role in the dissemination and transmission of this virus is not yet apparent but the observed facts are entirely compatible with the theory that the alimentary canal is the portal of entry and exit.

Immunity. Poliomyelitis is an example of a virus disease to which a healthy, well-nourished host is more susceptible than is a sickly, malnourished one (p. 223). The early clinical observers remarked that strong well-favored children were usually attacked by this disease. Experiments with the Lansing mouse-adapted strain and with Theiler's mouse encephalomyelitis (p. 910) show that well-nourished animals are more susceptible to the virus. The first reports of this work indicated that a deficiency in vitamin B_1 was responsible for resistance, and further investigations[204] indicated that an overall restriction in the diet with adequate B_1 would give similar results.

Active Immunity. Second attacks occur in man and may be induced experimentally in the monkey. Considering the low incidence in the general population of recognizable poliomyelitis, it becomes difficult to demonstrate statistically that one attack produces any immunity against a second. When a convalescent monkey is reinoculated, extension of the infection in the central nervous system with or without paralysis appears to depend upon how much of the central nervous system was invaded following the first inoculation and whether a different strain of virus was used for the second exposure. It has been demonstrated that different neuron systems are attacked with subsequent injections by different routes.[205] Nevertheless a severe attack of the disease in a monkey usually renders it resistant to further extension of paralysis following a second injection with homologous virus.

Virus neutralizing substances have been found in the blood of recovered cases (human and monkey) and also in the blood of a large proportion of persons with no history of the disease. This last is taken as evidence of widespread subclinical infection and is not surprising in view of the high percentage (approximately 75 per cent) of unrecognized infections among intimate contacts of cases, as demonstrated by tests for virus in the stool (p. 907). The incidence of neutralizing sera by age groups increases with age in much the same manner as positive Schick tests in unimmunized populations. Other observations, however, have made the significance of the neutralizing property more doubtful. Some workers have found a considerable proportion of monkey and human convalescent sera devoid of this property and others have found neutralizing serum present at the onset of an attack. It must be remembered in this connection that the neutralization test as performed in monkeys is only very crudely quantitative and because the techniques have varied in different laboratories, optimum conditions for the demonstration of neutralizing property have

---

[203] Trask, Paul and Melnick: Jour. Exp. Med., 1943, 77:531.

[204] Foster, et al.: Jour. Exp. Med., 1944, 79:221; Rasmussen, et al.: Jour. Inf. Dis., 1944, 74:41.

[205] Howe and Bodian: Jour. Exp. Med., 1941, 74:145.

certainly not always been present. In addition, antigenic differences between strains may account for some of the negative results.[206] It was hoped that more carefully quantitated neutralization tests performed in mice with mouse-adapted strains of virus would solve the problem, but such is not the case.[207] Morgan[208] has shown in monkey poliomyelitis that the appearance of serum antibody is greatly delayed in paralyzed convalescent monkeys, but that neutralization can be accomplished early with extracts of susceptible grey matter. Unfortunately, at present there is no satisfactory test for detecting the immune state in man, or for studying his response to natural exposure.

Active Immunization. Experiments with monkeys have shown that crude virus or preparations treated in various ways will induce an immunological response when given by intradermal, subcutaneous or intramuscular routes. Only an occasional monkey succumbs to the disease from this procedure, and serum antibody appears in the survivors. There are claims that such injections render the monkeys resistant to inoculation of the virus into the central nervous system. Others have found that such monkeys remained susceptible to intranasal or intracerebral inoculation.[209] The production of neutralizing serum after artificial immunization of monkeys by the procedures that have been tried therefore does not have the same significance as it does in the case of some other infections, *e.g.* equine encephalomyelitis (p. 895). Attempts to immunize children were made with two types of preparation in 1935 and the evidence seems clear that there were twelve cases of poliomyelitis induced in children by the inoculation of vaccine containing active virus.[210] Studies on the serum of inoculated children failed to indicate clearly that inactivated vaccine induced an immunological response, and the use of these preparations was soon discontinued.

Experiments[211] with rodent strains of poliomyelitis indicate that exposure of virus emulsions to ultraviolet light destroys the infectivity but preserves the antigenicity. Most mice were protected against hundreds or thousands of MLD's by previous injections with such vaccines.

Passive Immunization. Attempts to prevent poliomyelitis in monkeys by prophylactic inoculation of convalescent or immune serum, or to modify the infection by treatment with serum after injection of the virus, have been almost entirely unsuccessful. The intracellular habitat of the virus has usually been offered as the reason for the failure of immune serum, present as the result of either active or passive immunization, to arrest the progress of the disease in monkeys. In the experimental monkey, direct contact with central nervous tissue afforded by intracerebral inoculation, and with olfactory nerve endings by intranasal inoculation, allows the virus to reach susceptible cells without being exposed to antibodies present in the serum.

[206] Stimpert and Kessel: Amer. Jour. Hyg., 1939, 29, Sect. B:57.

[207] Hammon, Mack and Reeves: Jour. Immunol., 1947, 57:285.

[208] Morgan: Amer. Jour. Hyg., 1947, 45:390.

[209] Kramer, *et al.:* Jour. Immunol., 1937, 31:167, 183, 191, 199; Olitsky and Cox: Jour. Exp. Med., 1936, 63:109; Gordon, Hudson and Harrison: Jour. Infect. Dis., 1939, 64:241.

[210] Leake: Jour. Amer. Med. Assn., 1935, 105:2152.

[211] McKinstry and Reading: Jour. Franklin Inst., 1944, 237:71; Milzer, Oppenheimer and Levinson: Jour. Amer. Med. Assn., 1944, 125:704.

In spite of the negative experiments with monkeys, convalescent serum has been used for the treatment of human cases, but various controlled observations fail to show any value in serum treatment.

Encephalomyelitis of Mice. In 1934 Theiler[212] reported the recovery from the intestines of normal laboratory mice of a neurotropic virus (TO) with many characteristics of the strains of poliomyelitis isolated from man. Since then a similar but not identical strain (FA) has been encountered. Although sometimes called "mouse poliomyelitis" these strains should not be confused with the rodent-adapted strains of poliomyelitis which originated in man.[213]

<div align="center">

RABIES (HYDROPHOBIA)[214]

</div>

Although the virus of rabies has a broad host range, all mammals and some birds being considered susceptible, rabies is primarily a disease of dogs, cats and wild carnivores. The bites of these animals account for most cases in other mammals and in man,[215] the virus being present in the saliva of rabid animals. Although only about 30 human cases of rabies are reported each year in the United States, a large number of canine cases are recognized and the control of this disease is an important public health problem.[216]

The Disease in Dogs. Rabies in the dog has a variable incubation period, cases occurring from ten days to six months after exposure. Almost half of the cases, however, have an incubation period of less than thirty days. In the initial stages the dog may show behavior changes and a perverted appetite. A period of increasing excitability may manifest itself first by signs of uneasiness and restlessness, progressing to a stage where the dog runs aimlessly about, snapping at objects in its path or responding with the greatest excitement and fury (furious rabies) to minimal stimuli. Beginning paralysis results in drooling of saliva due to inability to swallow, or the gait may be abnormal. As paralysis increases the dog becomes inactive and stuporous (dumb rabies) and death occurs.

The Virus. Virus is present in the central nervous system of affected animals, in the salivary glands and saliva, and inconstantly in other glands and in the blood. During the incubation period the dog is not infectious, *i.e.*, virus is not present in the saliva, until a few days (two to eight) before characteristic signs of disease appear. Thus a period of observation of fourteen days is sufficient after a dog has bitten to determine whether it was infectious at the time of biting. If signs of rabies do not occur within this period it is safe to conclude that no virus was present in the saliva at the time of biting.

Fixed Virus. The production of "fixed virus," a term applied by Pasteur to virus that is so exalted in virulence for rabbits by successive intracerebral passages that it will produce the death of these animals in six or seven days, is one of the classical examples of variation of a virus (p. 851). Such strains also

[212] *Cf.* Theiler: Jour. Exp. Med., 1937, 65:705.
[213] Melnick and Riordan: Jour. Immunol., 1947, 57:331.
[214] See review by Johnson: Ann. New York Acad., Sci., 1947, 48:361.
[215] McKendrick: Bulletin of the Health Organization, League of Nations, 1940, 9:31.
[216] Report of a committee of New York Academy of Medicine: Pub. Health Repts., 47, 62:1215.

have greatly reduced ability to invade tissue other than the central nervous system, and usually do not induce the formation of Negri bodies.

The virus of rabies is one of the larger viruses and is passed through bacteria-retaining filters only with difficulty. It has been propagated in tissue culture and in the nervous tissue of chick embryos.[217] It is said to be quickly destroyed when exposed to air and sunlight; drying from the frozen state preserves it for long periods. It is destroyed by many chemical disinfectants but is apparently sufficiently resistant to ether to allow this reagent to be used for the destruction of bacteria in putrefying brain tissue to be tested for the presence of virus.[218]
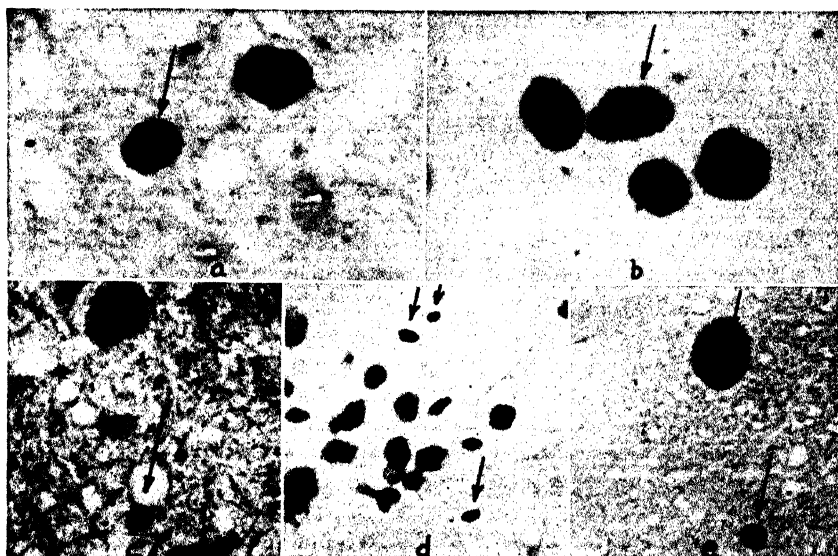
Fig. 257. Negri bodies in smear from infected dog brains. Sellers stain. Photomicrographs *a* and *b* were taken with a filter to bring out the granular nature of the inclusions, and *c, d,* and *e* similarly to contrast with the tissue cells. Reduced from the following magnification: *a* and *b:* × 1900, *c, d, e:* × 800. (From slides supplied through the courtesy of Dr. Joseph Zichis.)

Transmission to Man. It is very difficult to estimate the percentage of persons who, not receiving specific prophylaxis, develop rabies after the bite of a rabid animal. The figure has been estimated to be 9 per cent, but the likelihood of rabies developing in a given individual depends upon several factors. A severe wound is more liable to result in infection than a superficial one, but contact of saliva with freshly abraded skin is usually regarded as potentially dangerous. Bites made through heavy clothing are less likely to be followed by rabies than those on bare skin. Virus deposited at the site of a bite reaches the central nervous system by migration along peripheral nerves. This mechanism of pathogenesis doubtless accounts for the relation between

[217] Dawson: Science, 1939, 89:300.
[218] Sulkin and Nagle: Jour. Lab. Clin. Med., 1939, 25:94.

site of the bite and length of incubation period, which is longer with bites upon the extremities presumably because the virus has further to travel. The incubation period in man averages six to eight weeks but varies considerably and may be less than a month in the case of bites about the head and neck. Delay in beginning treatment increases the likelihood of a fatal outcome and this is especially true following bites about the head.[215]

Laboratory Diagnosis. Inflammatory and degenerative changes have been described in the central nervous system of rabid animals, especially in the spinal ganglia, but the most significant effect is the appearance of inclusion bodies named for Negri,[219] who first described them. Although there is no agreement concerning the essential nature of these bodies they have served as a valuable aid in the diagnosis of the disease. They are usually present in the brains of animals dying from infection with "street virus," but are not found in the brains of rabbits infected with "fixed virus." This is ascribed to their failure of development during the shorter incubation period of the latter infection. Smears of brain tissue, or "touch" preparations made by application of a slide to the cut surface of the brain, especially the hippocampus major and the cerebellum, are recommended for the detection of Negri bodies (Fig. 257). These may be stained by any one of several methods; that of Sellers[220] appears to have some advantages over previous methods. Negri bodies are acidophilic masses, usually spherical or oval in shape, seen in sectioned tissue to be within the cytoplasm of large ganglion cells (Fig. 233), and in smears often to be freed from disintegrated cells (Fig. 257). An internal structure can often be seen consisting of basophilic elements within the acidophilic ground substance. Since Negri bodies are not always demonstrable in brains later proved to contain rabies virus (Leach[221] reports that 12 per cent of brains negative for Negri bodies proved to be positive by animal inoculation), the laboratory diagnosis of rabies also involves inoculation of brain tissue into animals. Rabbits or guinea pigs have been used in the past, but there seems to be a distinct advantage in the use of mice, which have been shown to be regularly susceptible to intracerebral inoculation. After inoculation with rabies-positive brain material most mice die within ten to twelve days and Negri bodies can be demonstrated in some cases as early as the fifth or sixth day after inoculation.[220]

Rabies Prophylaxis. Pasteur's original method of rabies prophylaxis was to inoculate the exposed person daily with emulsions of rabbit spinal cord, infected with fixed virus, beginning with cord which had been dried for fourteen days and gradually increasing the strength of the dose by using cords which had been dried for shorter and shorter periods. Other methods of using gradually increasing doses of active virus have been introduced as well as the use of virus inactivated by various agents[222] (phenol, chloroform, ether, yatren, dialysis against distilled water, heat, ultraviolet light, antiserum). The com-

[219] Negri: Ztschr. f. Hyg., 1903, 43:507.

[220] Sellers: Amer. Jour. Pub. Health, 1927, 17:1080.

[221] Leach: Amer. Jour. Pub. Health, 1938, 28:162; see also Willett and Sulkin: Jour. Amer. Vet. Med. Assn., 1939, 95:659.

[222] Greenwood: Bull. Health Org., League of Nat. 1945–46, 12:301.

monly used Semple vaccine, inactivated by phenol, is injected daily for fourteen days. Similar preparations are used in the immunization of dogs.

Webster,[223] in a survey of the field, found that there was little satisfactory experimental evidence regarding the relative efficacy of these various preparations. This is evidently due to the difficulty of obtaining a regular response in animals to a test dose. Based on earlier work by Webster, the Habel test[224] for rabies vaccines has resulted in greater uniformity in commercial products. A vaccine prepared by short exposure of virus emulsion to ultraviolet light of high intensity has given evidence of superiority in comparative tests.

So-called paralytic accidents, of obscure etiology, sometimes follow prophylaxis against rabies. These occurred at the rate of 1 in 7250 treatments at the Pasteur Institutes[215] and there were 7 deaths in 42 cases. This condition, although rare, is a sufficient hazard to avoid giving prophylaxis to persons who have had no real exposure, either to allay their fears or to "play safe."

It has been recommended that prophylactic inoculation be given immediately under any of the following conditions:* (1) The animal was apprehended and presented clinical signs of rabies; (2) the animal was killed and the brain found positive for rabies, by microscopic examination; (3) the animal was killed and, though the brain was negative by microscopic examination, the animal was suspected of being rabid; (4) the person was exposed by a stray animal which escaped or could not be identified.

Rabies prophylaxis, although of great value, has the limitation of unsatisfactory protection when the incubation period of the disease is short. From work with dogs and from data on human rabies, it is concluded that little protection is afforded during the first month after exposure. The possibility of passive prophylactic immunization, after exposure, has been investigated by Habel.[225]

Although in most countries nearly all cases of rabies are caused by dogs or their near relatives, in some localities rabies has become established in and transmitted by other animals. Such is the vampire bat rabies of Mexico and parts of South America where it has become a serious veterinary problem, producing the paralytic form of the disease. Vampire bats, in contrast with other animals, can remain carriers over long periods of time without showing signs of illness.

OTHER VIRUS INFECTIONS OF MAN

Molluscum Contagiosum. This is a contagious disease characterized by superficial benign epithelial tumors of the skin of pearl-like appearance and with a small central depression. The filterability of the active agent appears to be established and histological study[226] indicates that the characteristic inclusion body is composed of elementary bodies in the same manner as those of fowlpox, etc. No susceptible experimental animal has been found.

---

* Quoted from Johnson[214] with permission of author and publisher. See also Sellers: Am. Jour. Trop. Med., 1948, 28:453.
[223] Webster: Amer. Jour. Hyg., 1939, 30:113.
[224] Habel: Pub. Health Repts., 1940, 55:1473.
[225] Habel: Pub. Health Repts., 1945, 60:545.
[226] Goodpasture and King: Amer. Jour. Path., 1927, 3:385.

Warts.[227] Benign epithelial tumors of several species of animals are known to be filterable. Infectious papillomas of rabbits have been mentioned in connection with other filterable tumors (p. 838). Papillomas of dogs and cattle and warts (verruca vulgaris) of man are reported to be filterable. Warts of various kinds contain intranuclear inclusion bodies.

Chickenpox (Varicella) and Herpes Zoster. These are regarded as due to virus agents although the evidence is not complete. Chickenpox is a highly infectious disease of children, usually mild in nature, and characterized by a vesicular eruption. No suitable experimental animal has been found, so that investigation has been handicapped. Elementary bodies, agglutinable in convalescent's serum, have been described in vesicle fluid.

Herpes zoster manifests itself by the appearance of dermal vesicles which follow the distribution of one or more sensory, cutaneous nerves, usually of the torso. Animal experiments with this infection have likewise been negative, although human transmission has been accomplished. Complement fixation with convalescent serum has been reported, using crusts of the dried lesions or vesicle fluid as antigen. Paschen in 1933 described the presence of elementary bodies in the vesicles.

Although these two maladies have little in common clinically, an epidemiological association between the two has been noted for many years. Herpes zoster may appear in a person following contact with a case of chickenpox, but more often cases of the latter have been seen to follow contact with herpes zoster. Evidence to explain this association has recently been found in the laboratory. It is claimed that antigens made from the lesions of either disease will fix complement in the presence of convalescent's serum from either herpes zoster or varicella. Amies[228] employed an agglutination technique with pure suspensions of the elementary bodies of both diseases against convalescent's sera from both sources. Some cross-agglutination was obtained but the reactions were not so good as with homologous serum and elementary bodies. The results were interpreted as showing a degree of antigenic similarity between the two agents.

Epidemic Keratoconjunctivitis. During 1941 and 1942 outbreaks of this eye infection made their appearance in the United States following probable importation of the disease from Hawaii. Although occurring in large numbers in shipyard workers on the West Coast,[229] this feature apparently has no relation to etiology. The disease is characterized by a high degree of infectiousness, relatively little ocular exudate, frequent swelling of the regional lymph nodes, and systematic symptoms, especially headache. The incidence of complications producing impairment of vision has been variable. Sanders[230] successfully isolated a filterable agent in tissue culture in 1942 and was later able to infect mice. The demonstration of neutralizing antibody for this agent in convalescent human sera and the production of a mild case in a human

[227] Findlay: Chap. 18 (p. 252), *A System of Bacteriology,* Vol. 7. H. M. Stationery Office, London. 1930.

[228] Amies: Brit. Jour. Exp. Path., 1934, *15:*314.

[229] Rieke: Jour. Amer. Med. Assn., 1942, *119:*942.

[230] Sanders: Jour. Exp. Med., 1943, 77:71.

volunteer with cultured virus, fulfilling Koch's third postulate, provide good evidence that the agent isolated is the cause of the disease.

Rift Valley Fever is a disease of sheep and cattle present in East Africa, probably mosquito-borne. Cases have been noted in sheepherders during epizootics and several laboratory infections have occurred.[231] It is described as an influenza-like or dengue-like disease and the virus is present in the blood. Neutralization tests with the sera of natives and Europeans in Africa indicate that the infection is not confined to Kenya, where it was first described, but is present also in other parts of East Africa. The suspicion, based upon indirect evidence, that mosquitoes transmit the disease has been confirmed by isolation of virus from mosquitoes caught in the Semliki Forest.[232] Laboratory transmission by mosquitoes is also reported.

Foot-and-Mouth Disease. This infection of cattle is apparently able to attack man. Although many cases have been suspected, only a few have been definitely proved by animal inoculation and examination of serum after recovery. The characteristic lesion in animals is a vesicle upon the skin or mucous membranes, and in the proved cases in man transient vesicles were present upon the hands and feet.

Newcastle Disease of poultry, recently recognized in the United States, is apparently able to cause conjunctivitis in man. A laboratory accident in Australia provided the first evidence for this, and an outbreak reported from Palestine[233] among women who had handled infected poultry was interpreted on epidemiologic grounds as caused by the infected chickens.

Fort Bragg (Pretibial) Fever.[234] This is an acute febrile disease, often with an erythematous rash over the pretibial regions, recently recognized as a viral infection. Hamsters are used for propagation in the laboratory and growth in embryonated eggs occurs. Inoculated chimpanzees developed an illness similar to that seen in man.[235]

Colorado Tick Fever.[236] This disease is transmitted by the tick, *Dermacentor andersoni*, and has been recognized only in the Rocky Mountain region of the United States. It is a generalized febrile illness characterized by periods of attack and remission. Although there is striking similarity to dengue fever, the two diseases are immunologically distinct. The agent is found in the serum of infected hamsters and has been estimated by filtration experiments to have a size of approximately 10 mμ, making it one of the smallest viruses. It has been adapted to the mouse brain and to chick embryos.[237]

[231] Francis and Magill: Jour. Exp. Med., 1935, 62:433.
[232] Smithburn, Haddow and Gillett: Brit. Jour. Exp. Pathol., 1948, 29:107.
[233] *Cf.* Burnet: Med. Jour. Austral., 1943, 30:313; Yaton: Harefuah, 1946, 30:57.
[234] Daniels and Grennan: Jour. Amer. Med. Assn., 1943, 122:361.
[235] Melnick and Paul: Proc. Soc. Exper. Biol. Med., 1948, 67:263.
[236] Florio and Stewart: Amer. Jour. Pub. Health, 1947, 32:293.
[237] Koprowski and Cox: Jour. Immunol., 1947, 57:255.

BACTERIOPHAGE[1]

Transmissible lysis of bacteria was discovered by Twort in 1915 and rediscovered by d'Herelle in 1917, and is generally known as the *Twort-d'Herelle phenomenon*. The lytic agent which brings about the dissolution of the bacterial cells is known as *bacteriophage*, or, more briefly, as *phage*. The lysis is transmissible indefinitely in series, and the lytic agent is filterable through the usual bacteria-proof diatomaceous earth filters. The resemblance of this trans-
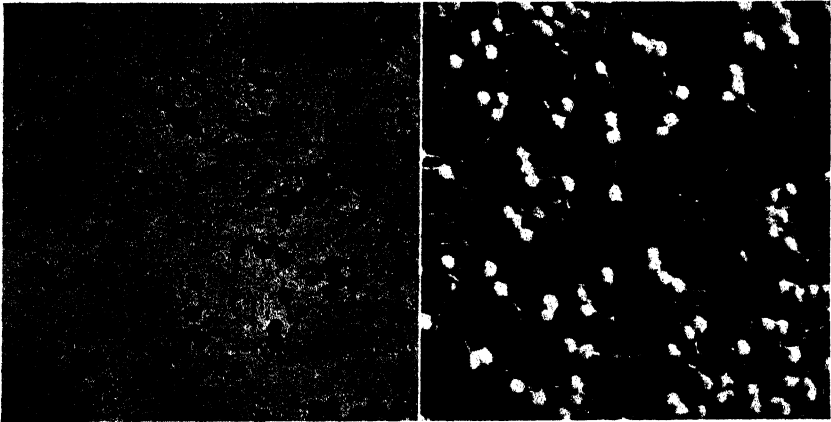
Fig. 258. Electron micrographs of coliphage, strain gamma. The phage was washed by centrifugation, suspended in saline and diluted with 0.025 M $CaCl_2$. The plate on the left shows untreated phage, and that on the right a similar preparation shadow-cast with chromium. The shape of the particles is slightly altered and the surfaces somewhat roughened by the washing. (Sharp, SAB No. 139.)

missible lysis of bacteria to the virus diseases is striking, and the Twort-d'Herelle phenomenon may not unreasonably be regarded as a filterable virus disease of bacteria and the phage as the virus.

Demonstration of Bacteriophagy. Bacteriophage may be separated from contaminating microorganisms by filtration through Berkefeld N or similar filters. When a drop of the phage-containing filtrate is added to a young broth culture of susceptible bacteria, within an hour or two the culture clears, few if any bacteria can be found on microscopic examination, and those which

[1] See the following reviews: Hadley: Jour. Inf. Dis., 1928, 42:263; d'Herelle: *Bacteriophage and Its Clinical Applications*. Charles C Thomas, Springfield. 1930; Burnet: Biol. Rev., 1934, 9:332; Delbrück: Advances in Enzymology, 1942, 2:1; *ibid.*, Biol. Rev., 1946, 21:30.

are present may be the poorly staining "ghost cells." The lysed culture may be filtered and, with the addition of the filtrate to a fresh young broth culture, the phenomenon is repeated and may be continued indefinitely.

Bacteriophage may be found in a variety of places in nature, notably feces and sewage. Filtrates from fecal suspensions or sewage do not always show pronounced lytic activity per se, but if the bacterial culture to which the filtrate has been added is filtered in turn, and a drop of that filtrate added to a fresh broth culture, etc., complete lysis will appear after a few such "transfers." Filtrates from lysed cultures are generally of high potency and may be diluted to $1:10^9$ or $1:10^{10}$ and still bring about lysis in 1 ml. quantities.

The lytic activity of bacteriophage may also be shown on solid media. If the surface of nutrient agar upon which there is a uniform growth of young bacterial culture is streaked with an inoculating needle which has been dipped
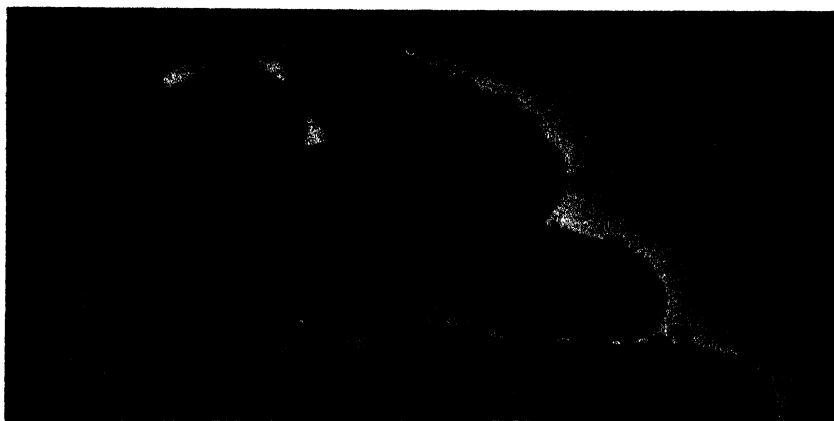
Fig. 259. *Bacterium coli* and coliphage after ten minutes' incubation. Note the adsorption of the phage bodies on the surface of the bacterial cells. Electron micrograph; × 17,000 (Luria and Anderson).

in phage, clearing of the culture along the needle tract becomes apparent within a few hours. If similar bacterial cultures in Petri dishes are covered with successive dilutions of phage, there will be clearing over the entire surface in the lower dilutions, more dilute phage produces a moth-eaten appearance, and highly diluted phage produces discrete cleared areas in the bacterial growth. These cleared areas, or *plaques*, are analogous to bacterial colonies and the phage may be picked from such "colonies" by touching with an inoculating needle and transferring to a fresh young culture of susceptible bacteria. The plaques are variable in size but constant for a given strain of phage; some phages are "large plaque phages" and others are "small plaque phages."

Characteristics of Bacteriophage. That bacteriophage is particulate is indicated by the formation of plaques, and quantitative studies have shown that the number of plaques decreases in linear relation to increasingly higher dilutions of phage-containing filtrates. Similarly, if equal amounts of phage diluted to almost the limit of activity are distributed in a series of bacterial cultures, the proportion of tubes showing lysis is what would be expected if the lytic
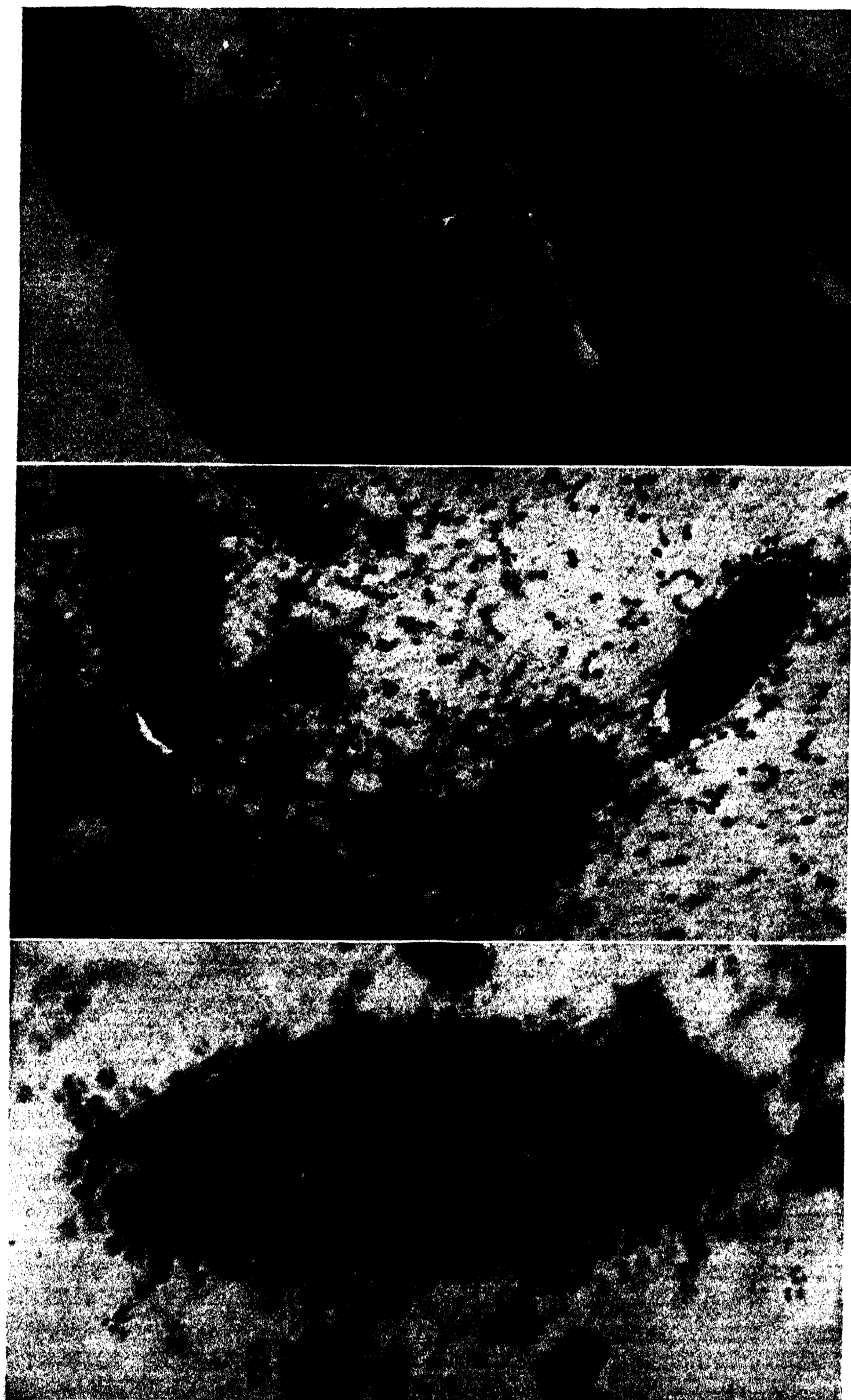
Fig. 260.

agent were particulate. Phage, then, does not exist in true solution. The size of the particles has been measured by filtration through gradocol membranes and by high-speed centrifugation. Pure phages are found to be highly uniform in size; different phages range in continuous series from 8 to 12 mμ to 50 to 75 mμ in diameter. Examination of electron micrographs of phage and phage lysis has shown that some phage particles tend to be cubical in shape, while others are round and in some cases are characterized by the presence of a "tail," giving an appearance somewhat similar to spermatozoa.[2] The tail is not an organ of locomotion, however, for it does not contribute to the rate of diffusion of the phage.[3]

Multiplication. Because the activity is indefinitely transmissible in series, it will be clear that the phage particles increase in number, and it may be observed that this increase is coincidental with the actual process of lysis. Such multiplication occurs, however, only at the expense of living bacterial cells; phage will not increase in absence of susceptible bacteria or in the presence of dead bacteria, though it may be shown that phage is absorbed by the dead cells of the susceptible bacterial species. It is generally agreed that multiplication of phage requires the presence of young, actively growing and metabolizing bacteria; it does not occur to any great extent in old cultures or in washed suspensions of "resting bacteria" in non-nutritive buffer solutions.

It was early suggested by d'Herelle that a phage particle enters a susceptible bacterial cell, multiplies and, with the dissolution of the cell, a considerable number of particles are liberated. Such a process has, in fact, been observed directly. In keeping with this, it is found that the phage titer in a lysing culture increases in steps, the number of phage particles increasing by increments varying from 20 to perhaps 1000; Delbrück[4] speaks of this increment as the "burst size." As lysis proceeds, however, the rate of increase is observed to become logarithmic, as might be expected when the individual cellular dissolutions get out of step with one another and thus cancel out the step-like character of the increase.

Specificity. The bacteriophages are specific in their action, *i.e.*, with respect to the kind of bacteria that are susceptible to lysis. In some instances a relatively broad range of activity may be observed and more than one species of bacteria may be lysed by a single phage; in other cases the activity is highly specific and only a single strain may be lysed. It has been shown, largely

[2] Luria, Delbrück and Anderson: Proc. Nat. Acad. Sci., 1942, 28:127; Jour. Bact., 1943, 46:57.

[3] Polson: Proc. Soc. Exp. Biol. Med., 1948, 67:294.

[4] Delbrück: Jour. Bact., 1945, 50:131.

Fig. 260. Electron micrographs showing the lysis of *Bact. coli* by coliphage T-2. The top plate shows a single lysed cell together with normal cells produced in thirty minutes after inoculation of the bacterial growth on collodion film with dilute phage containing a calculated single phage particle per bacterial colony. Note the phage particles within the cell. × 15,200. The center plate is a similar preparation after eighteen hour incubation, showing large numbers of phage particles among the cellular debris and two unlysed bacterial cells. There appears to be no evidence of division of phage particles. × 14,400. The bottom plate illustrates the kind of cell often found after eighteen-hour incubation in which the cell substance of the bacterium is completely replaced by a dense mass of phage particles. × 40,000. (Mudd, Hillier and A. G. Smith.)

through the work of Burnet, that phage specificity is associated with the anti-genic structure of the bacteria. This is indicated directly in the case of certain Salmonella species; those with common somatic antigens are lysed by a single phage. It is also shown indirectly in the specific inhibition of phage lysis. It has been found that extracts of bacterial cell substance will specifically inhibit lysis; these extracts are inactivated by the homologous antibacterial serum and a pre-cipitin reaction occurs between the two. The precipitating antigen is identical with the phage-inhibiting substance, and the specificity of this substance is determined by a polysaccharide haptene. This haptene, it may be noted, is in-effective in purified form. The antigenic structure of the host bacterium is not, however, the only element in specificity of phages, for phage-resistant variants may have the same antigenic structure as the parent susceptible strain.
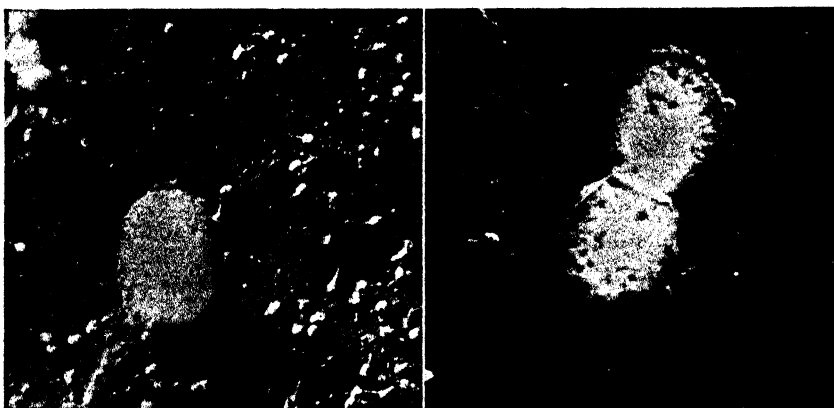
Fig. 261. Electron micrographs of shadow-cast preparations showing the lysis of *Bact. coli* by coliphage T-4. That on the left shows an intact bacillus with surrounding and "adsorbed" particles of phage. $\times$ 15,000. The right shows a bacillus about to undergo fission from which the cell membrane has begun to peel away. $\times$ 20,000. (Wyckoff.)

Metabolism. Since phage multiplies only in the presence of actively metabolizing bacteria, studies on the physiological activity of phage itself are difficult. Attempts have been made, however, to detect· evidences of the metabolism of phage in the absence of bacteria. Bronfenbrenner was unable to detect the evolution of carbon dioxide from filtrates containing phage in high concentration in a specially devised microrespirometer over a period of ten days, and concluded that if respiration occurs it must be at least 10,000 times slower than the respiration of bacterial spores. The same worker showed that, when appropriate corrections were made, the oxygen uptake of lysing cultures was not greater than that of control cultures. Other studies on the reducing power of bacterial cultures have indicated that this ability is not affected in such a way as to indicate a physiological activity on the part of the phage. It appears, therefore, that bacteriophage does not metabolize to a de-tectable extent in ordinary ways.

There is some evidence, however, that phages have certain "nutritive re-quirements." Spizizen[5] found that certain amino acids, especially glycine,

[5] Spizizen: Jour. Inf. Dis., 1943, 73:222.

nucleic acids, and ferric, ferrous, magnesium and manganese ions markedly stimulated phage production in the absence of bacterial multiplication. Similarly, Anderson[6] has found that tryptophane and some related compounds, and to a lesser extent phenylalanine, are necessary for the initial adsorption of some, but not all, coliphages. Fitzgerald and Babbitt[7] have shown that compounds of the acridine series give an inhibition of phage production that cannot be accounted for by bacteriostatic effects, and the inhibition is counteracted by ribose nucleic acid. Such observations need not be interpreted as indicating an independent metabolism on the part of phage, but rather suggest that more or less specific metabolic reactions of the host bacteria are concerned with the adsorption of phage particles and their generation within the infected cell.

Phage is but little more resistant to bactericidal chemicals and physical influences such as irradiation than the vegetative cells of bacteria. Lytic filtrates may be preserved in the refrigerator for a year or more without significant loss of titer, though the activity of such filtrates shows marked deterioration when stored at room temperature over long periods of time. Some phages are unable to develop in the presence of citrate, *i.e.*, in the absence of calcium, but others are not so affected. Some phages are inactivated by urea.

Chemical analyses of purified phage (coliphage T–2) have indicated that essentially the same constituents are present as in bacteria and the larger viruses, *viz.*, protein, lipid, carbohydrate and nucleic acids. Taylor[8] has reported that the lipid is neutral fat, lacking phospholipid and sterols, and the nucleic acids consist of desoxypentose and ribopentose nucleic acids, the former predominating. In analyses of this same phage Cohen and Anderson[9] have found phospholipid, but no ribose nucleic acids.

Mutations. Though they appear to have no independent metabolism, the phages, like other viruses, show mutation-like changes characteristic of living organisms. Earlier studies on adaptive changes are not too significant since mixed phages were frequently used and an apparent adaptive change may arise from selection. Luria[10] has found that mutation-like changes in the specificity of phage for host bacterium may occur, however, which breed true. Similarly, Hershey[11] has observed a mutation-like change with respect to size and type of plaque formed which breeds true and is not associated with any change in immunological or host specificity.

Antigenicity. The phages are antigenic, and the sera of animals immunized in the usual way with filtrates specifically inhibit the lytic activity. There is also evidence that the phage particles are agglutinated by such antisera. The antisera also contain antibodies to the bacterial substance present in filtrates from lysed cultures. The phages are immunologically heterogeneous, but a number of distinct groups are apparent; it is of some interest that members of the same group may be found in localities geographically widely separated.

There appears to be some correlation between immunological type and the

6 Anderson: Jour. Cell. Comp. Physiol., 1945, 25:17.
7 Fitzgerald and Babbitt: Jour. Immunol., 1946, 52:121, 127.
8 Taylor: Jour. Biol. Chem., 1946, 165:271.
9 Cohen and Anderson: Jour. Exp. Med., 1946, 84:511.
10 Luria: Jour. Bact., 1944, 47:416.
11 Hershey: Genetics, 1946, 31:620.

ability to develop in the absence of calcium, resistance to urea, resistance to irradiation, etc., in that the phages belonging to one immunological group exhibit much the same response to such agents but may differ widely in this respect from the more or less homogeneous response of a different immunological group. In the current edition of Bergey (1948) an attempt is made to classify the phages as species of the single genus Phagus on the basis of host specificity and plaque size.

Mechanism of Phage Action. The sequence of events following the introduction of phage into a culture of susceptible bacteria is divisible into three stages, the adsorption of phage particles on the surface of the bacterial
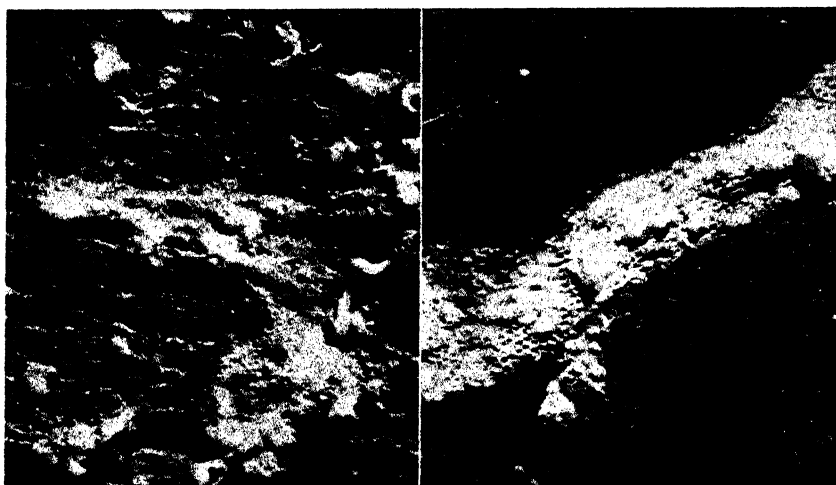
Fig. 262. Electron micrographs of shadow-cast preparations of *Bact. coli* lysed by coliphage. The micrograph on the left shows the filamentous protoplasm; structure can be seen in the filaments just above the central mass, and the structure forms a net in the mass at the bottom. The separate opaque pieces casting long shadows are fragments of bacterial cell membrane. × 15,000. On the right the longitudinal filaments and interweaving cross filaments are shown in an elongated bacillus undergoing lysis; note also the oblique net in the lower left corner. × 22,500. (Wyckoff.)

cell, the appearance of large numbers of phage particles within the cell, and finally the lysis of the infected cell to liberate the phage. This process is illustrated in the accompanying electron micrographs.

Adsorption. Adsorption of phage on the surface of the cell is highly specific and does not occur when the bacteria are not susceptible. It is at this stage that serum neutralization of phage activity occurs, for phage treated with antiserum is inactive, but antiserum is not effective once adsorption and presumably penetration of the host cell have occurred. Physiological factors are of significance at this point also; it has been pointed out earlier that adsorption of some phages will not occur in the absence of certain metabolites such as tryptophane (Anderson). Active metabolism of either host cell or phage, however, appears not to be essential, for phage inactivated by ultraviolet irradiation is adsorbed by susceptible bacteria, and phage is adsorbed on killed susceptible bacteria, though in neither instance, of course, is adsorption followed by intra-

cellular generation of phage and lysis. Krueger[12] has shown that the rate of adsorption is logarithmic, increases with rise in temperature, and proceeds until about 90 per cent of the phage has been adsorbed. The total phage remains relatively constant with, of course, a sharp drop in the free, *i.e.*, unadsorbed, phage.

Intracellular Generation of Phage. Following adsorption the phage particles presumably penetrate into the host cell though actual penetration of the intact cell wall has not been observed. During a period of latency, perhaps fifteen to twenty minutes with most phages, there is no increase in free phage, but a rise in total phage due to an increase in intracellular phage (Krueger). The
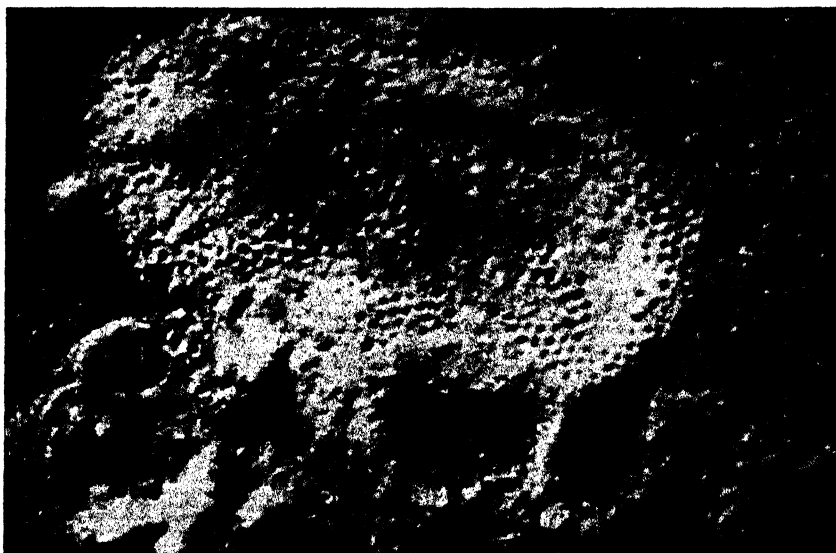
Fig. 263. Electron micrograph of shadow-cast *Bact. coli* lysed by coliphage T-3. Note the many fine fibers crossing one another in the central, non-honeycombed areas which appear to remain behind to form the walls of the net left by the emerging phage particles. × 37,800. (Wyckoff.)

manner in which phage is generated within the cell is completely unknown. Multiplication by fission has never been observed; rather the entire internal structure of the infected cell becomes altered and seemingly differentiated into masses of phage particles in a honeycomb structure of protoplasm which are arranged in orderly and symmetrical patterns of a geometrical regularity that is most marked with the "tailless" phages. This differentiation of the cell substance has been demonstrated by electron micrography by Wyckoff[13] and by Mudd, Hillier and Smith.[14] The number of phage particles contained within a single cell is usually 100 to 200. Some evidence suggests that a phage precursor occurs within the cell[15] and it has been suggested that some key enzyme

[12] Krueger: Jour. Gen. Physiol., 1946, 30:25.
[13] Wyckoff: Proc. Soc. Exp. Biol. Med., 1947, 66:42; Biochimica et Biophysica Acta, 1948, 2:27.
[14] Mudd, Hillier and Smith: Personal communication from Dr. Mudd.
[15] Krueger and Scribner: Jour. Gen. Physiol., 1939, 22:699.

system is involved.[16] It is a matter of some interest that more than one kind of phage cannot be generated simultaneously within a single host cell, an interference phenomenon analogous to that observed with other viruses. Futhermore, the phage which does parasitize and generate within the host cell is affected by the one that does not in that the number of phage particles formed is reduced. Delbrück[17] has termed the first an "exclusion effect" and the second a "depressor effect." He assumes that the first phage particle entering the cell is the infecting agent, and the membrane of the infected cell is rendered impermeable to other phages, a hypothetical explanation in that there is no unequivocal evidence that penetration occurs in the first instance.
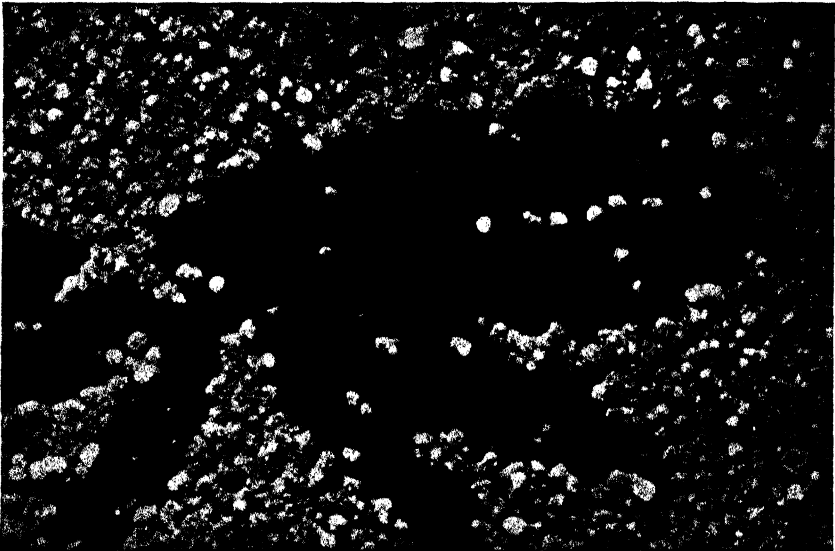
Fig. 264. Electron micrograph of a shadow-cast preparation of a phage plaque left by the lysis of *Bact. coli* by coliphage T-5. The granular texture of the fragments of bacterial protoplasm is clearly evident. × 19,440. (Wyckoff.)

Lysis. With maturation, so to speak, of the process of phage generation within the cell, the cell breaks down to liberate the phage particles, and the amount of free phage increases sharply in the characteristic step-like manner. There is some evidence of proteolysis at this stage, but it is difficult to know whether it results from incidental autolysis or is causally related to disintegration of the cell. Anderson[18] has found a bacteriolytic substance associated with purified phage that is liberated from the phage particles by sonic disintegration or ultraviolet irradiation. In any case, it seems clear that the cell structure of the bacterium breaks down with the liberation of phage and extrusion of the symmetrically patterned protoplasm noted above.

Effect of Phage on Bacteria. The most obvious effect of phage on susceptible bacteria is, of course, the lysis of the vast majority of the cells. Pre-

[16] Delbrück and Luria: Arch. Biochem., 1942, 1:111, 207.
[17] Delbrück: Jour. Bact., 1945, 50:151.
[18] Anderson: Jour. Coll. Comp. Physiol., 1945, 25:1.

liminary to lysis, however, there is a stimulation of growth of the bacteria; this effect is transitory and is very soon masked by the developing lytic process. On continued incubation of the lysed culture the few resistant cells remaining multiply with the production of a resistant strain of bacteria. This resistant strain may be unaltered in its immunological character and behavior toward other phages and may differ from the parent strain only in that it is not susceptible to lysis by that particular phage. In many cases, though, changes in other characteristics may accompany the acquisition of resistance; most commonly a certain degree of roughness is apparent in resistant strains derived from smooth parent strains. Phage lysis is a potent method of inducing bacterial variation, but the character, biochemical, serological, etc., of the variants cannot be predicted.

In this connection it is of interest that a symbiotic relation may exist between a phage and a resistant bacterium. Such a relationship is not uncommon, and phage-carrying strains of bacteria are termed *lysogenic*. The classic example of a lysogenic strain is a strain of *Bacterium coli* isolated by Lisbonne and Carrère[19] and sometimes known as coli-Lisbonne. Filtrates from broth cultures of this strain would regularly lyse Shiga dysentery bacilli. The relatively common occurrence of lysogenic strains of bacteria may be of considerable significance to the phenomenon of bacterial variation.

Therapeutic Use of Phage. d'Herelle has regarded the presence of phage as an important factor in recovery from infectious disease and has urged its therapeutic administration in a variety of infectious processes. The evidence does not, however, support the assertion that phage plays a part in recovery from infectious disease, and it is probable that it is of little or no significance. Phage therapy has been attempted in a variety of infections, including intravenous injection in septicemia, oral administration in enteric infections such as dysentery, and local application on boils and similar superficial abscesses, and instillation into the urinary bladder in cases of cystitis. The results have been disappointing, however, and it appears that phage therapy is of little value.[20] In some instances in which favorable results have been observed, equally favorable reactions have been secured with sterile broth or phage-free filtrates of bacterial cultures. The beneficial effects of the latter preparations have been ascribed by some to the specific immunizing action of dissolved bacterial cell substance.

[19] Lisbonne and Carrère: Compt. Rend. Soc. Biol., 1922, 86:569.

[20] *Cf.* Krueger and Scribner: Jour. Amer. Med. Assn., 1941, *116*:2160; Morton and Engley: Jour. Amer. Med. Assn., 1945, *127*:584.

INDEX

Extended discussions are indicated by page numbers in **bold-face type.**

A ANTIGEN, of Brucella, 495
    of coliform bacteria, 424
AP virus, 871
Abdominal-typhus, 453
Abiogenesis, 4
Ablastin, 285, 305, 733, 769
Abortin, 343, 497
Abortion, brucellosis and, 498
    contagious, in cattle, 496
Abrin, 203, 206
Abrus precatorius (jequirity bean), 206
Abscesses, Bact. coli and, 420
    Bacteroides and, 540
    Proteus and, 430
    Ps. pyocyanea and, 530
Absorption, agglutinin, 300
    mirror, of agglutinins, 300
Acanthocheilonema perstans, 814
Accelerated death phase, of growth, 57
Accelerated reaction, to smallpox vaccina-
    tion, 861
Accessory growth substances, 117
Accomplices, 215
Acetic acid, formation of, 94
Acetoin, 93
Acetyl glucosamine, in hyaluronic acid, 212
Acetylation of antigens, 280
Achorion, 655, 692
    A. gypseum, 700
    A. quinckeanum, 701
    A. schoenleinii, 681, 694, 701
Achromatium, 79
Achromobacteraceae, 194
Acid agglutination, 302
    curd, 12
Acid-fast stain, 19
Acidophilus milk, 534
Acids, effect of on bacteria, 145
Aciduric bacteria, 145, 531
Acquired character, immunologic, 182
Acquired immunity, 330-334
    methods of producing, 330
Acridine dyes, antibacterial action of, 149
Acriflavine, antagonized by nucleotides, 155
    antibacterial action of, 149
Actinobacillosis, 672
Actinobacillus, 672-673

Actinobacillus actinoides, 673
    A. actinomycetum-comitans, 673
    A. lignieresi, 672
    A. mallei, 601
Actinomyces, **656-676**
    A. albus, 129
    A. antibioticus, 129
    A. asteroides, 655, 668
    A. bahiensis, 668
    A. bovis, 655, 659-666
        cultivation of, 660
        morphology in culture, 661
        morphology in tissues, 660
        pathogenicity for animals, 663
        pathogenicity for man, 664
        physiology of, 662
    A. dermatonomus, 670
    A. farcinicus, 670
    A. graminis, 660
    A. gypsoides, 668
    A. hominis, 660
    A. israeli, 660
    A. keratolyticus, 669
    A. lavendulae, 129
    A. madurae, 655, 668
    A. minutissimus, 655, 670
    A. muris, 670
    A. muris-ratti, 670-672
        pleuropneumonia-like organisms
            and, 552
    A. necrophorus, 542
    A. rhusiopathiae, 673
    A. somaliensis, 668
    A. violaceus, 129
Actinomyces lysozyme, 129
Actinomycetaceae, 658
Actinomycetales, 194
Actinomycetes, **656-676**
    antibiotic substances from, 129
    classification of, 658
    cultivation of, 661
    differentiation of, 688
    morphology of, 657
    pathogenic, summary of, 659
    pathogenicity for animals, 663, 670
    pathogenicity for man, 664, 668-670
    physiology of, 662

927